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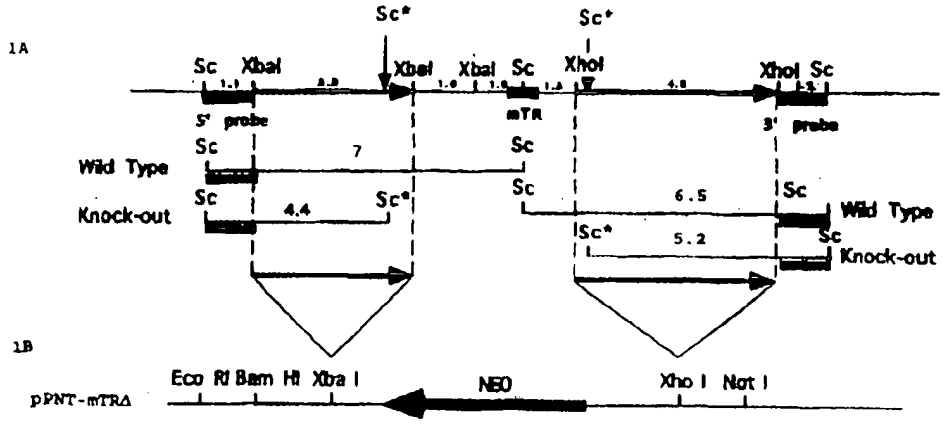

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(57) Abstract

Transgenic nonhuman organisms, such as transgenic mice, with altered expression of telomerase are provided. Methods of using these organisms, including methods of detecting compounds that affect the expression of telomerase are also disclosed.

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TRANSGENIC ORGANISMS WITH ALTERED TELOMERASE ACTIVITY

Background of the Invention

Telomerase is a ribonucleoprotein DNA polymerase that adds nucleotide sequence repeats to the telomeres of chromosomes as cells divide. Telomerases are comprised of essential RNA and protein components. Blasco, M.A., *et al.* (1995) *Science 269*:1267-1270; Feng, J., *et al.* (1995) *Science 269*:1236-1241; Collins, K., *et al.* (1995) *Cell 81*:677-686. Without telomerase, the telomeres of chromosomes shorten with each replication until they reach a critical length at which chromosome stability is affected.

Studies of telomerase suggest that the telomerase enzyme may be a new target for cancer therapy and a key to aging and the finite lifespan of somatic cells. Harley, C., *et al.* (1990) *Nature 345*:458-460. Kim, *et al.* (1994) *Science 266*:2011-2015, were not able to detect telomerase in a large number of primary cell lines and primary human tissues. When the number of telomeric simple sequence repeats reaches a critical minimum size, these cells become senescent. Harley, C., *supra*; Counter, *et al.* (1992) *EMBO J. 11*:1921-1929. Unlike most somatic cells, germline cells express telomerase and maintain telomere length throughout cell divisions.

Short telomeres are also found in immortalized cells, including human tumor cell lines. In contrast to normal human somatic cells, however, cancer cells from tissue culture and those taken directly from tumors contain detectable telomerase activity, suggesting the telomere length is maintained so these cells can divide indefinitely. Counter, *et al.* (1994) *PNAS 91*:2900-2904; Kim, *et al.*, *supra*. These findings suggest that targeting telomerase may be an effective cancer treatment. Harley,

et al. (1994) *Cold Spring Harbor Lab Symposium on Quantitative Biology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Because telomerase plays a key role in aging and human disorders, it is essential to understand how telomerase functions.

Summary of the Invention

This invention provides nonhuman transgenic organisms in which telomerase activity is altered. Organisms with one altered gene are heterozygous for the alteration; whereas, organisms in which the alteration occurs in both alleles are homozygous for the alteration.

In one embodiment, telomerase activity is reduced or eliminated in the nonhuman transgenic organisms because the gene or genes encoding one or more telomerase components is "knocked out" (*i.e.*, deleted or otherwise disabled), with the result that telomerase activity is reduced or eliminated.

In a second embodiment, nonhuman transgenic organisms contain nucleic acid constructs which induce telomerase activity by turning on endogenous genes encoding telomerase RNA and protein components that are normally silent, or through insertion of one or more nucleic acid constructs comprising DNA or RNA encoding telomerase RNA and/or protein components into the genome of an organism, in which they are expressed. Alternatively, an exogenous DNA construct, such as a promoter gene, is inserted into the genome of an organism and alters the normal transcription or functioning of endogenous telomerase. This promoter can be an inducible promoter, which is inserted and integrated into the genome of the organism so that endogenous or exogenous genes encoding telomerase are turned on or off at particular times and in selected tissues.

In a third embodiment, a nonhuman transgenic organism is provided wherein an endogenous telomerase component gene

is replaced, at least in part, with a telomerase component gene from another species or a telomerase component gene from another species which has been altered, thus producing an organism with a chimeric gene. The replacement can occur on both endogenous genes, resulting in an organism that is homozygous for the exogenous chimeric gene and is not capable of expressing any telomerase gene at that genomic location or is only capable of expressing the novel nucleotide sequence or transgene.

In another aspect, the invention provides a rodent, such as a mouse in which expression of the gene encoding a telomerase component is altered. In one embodiment, the endogenous gene is removed, partially or completely and, as a result, telomerase activity is reduced or absent. The rodent can be heterozygous or homozygous for the alteration. In a second embodiment, telomerase activity is induced by providing a DNA construct which turns on a normally silent endogenous gene. In a third embodiment, telomerase expression is altered by insertion, into the genome of the rodent, of a nucleic acid sequence that replaces all or a part of endogenous DNA encoding a telomerase RNA or protein component, the result of which is a chimeric telomerase molecule.

The nucleic acid sequence which replaces the endogenous gene encoding a telomerase component or a portion thereof can be a novel DNA sequence, a portion of the telomerase component gene, a marker gene, a promoter or other regulatory sequence, or a combination of these sequences.

The invention further provides constructs, particularly DNA constructs, useful for producing the transgenic nonhuman organisms, such as transgenic mice, described herein. Also provided are nucleic acid probes which can be used to distinguish DNA of a wildtype (naturally-occurring) organism from DNA of an organism in

which a portion of an endogenous telomerase component gene has been replaced with an exogenous DNA sequence. Further, nucleic acid constructs of transgenic unicellular eukaryotes such as *Tetrahymena sp.*, or in transfected prokaryotes are disclosed which are useful for production of telomerase or telomerase components.

In another aspect, this invention provides embryonic stem cells, somatic cells and tissues of a nonhuman organism which contain one or more copies of the nucleic acid constructs described herein. This includes cells and tissues comprising knockout constructs or constructs which induce telomerase expression. The transgenic organisms can be used as a source of cells for cell culture.

In a further aspect, the invention provides a method of identifying a drug for stimulating telomerase activity in a transgenic nonhuman organism, such as a mouse, with reduced telomerase activity. Alternatively, compounds that inhibit telomerase activity can be identified and/or tested for toxicity using a transgenic nonhuman organism to determine if telomerase can be inhibited or suppressed without detrimental effects to the organism. The drug can be administered to the organism and a sample of cells or tissues from the organism can be assayed for telomerase activity and for toxic side effects.

The invention further provides a method of using a transgenic organism or cells or tissues from the organism or its descendants to identify the control elements of immortal cells, such as cancer cells, or to identify the controlling factors in agents designated for anti-tumor or anti-aging purposes. In addition, agents that stimulate or restrict these phenomena can be identified and developed for prophylactic or therapeutic applications through the use of such transgenic organisms and their descendants.

Brief Description of the Drawings

Figures 1A and 1B diagram the targeting construct for knocking out the mouse telomerase RNA component. Figure 1A diagrams the portion of the wildtype mouse genome which includes the mouse telomerase RNA component (mTR). Plasmid pPNT-mTRΔ (Figure 1B) shows the vector used to replace the endogenous 3.9 kb chromosomal segment including the mouse gene for the telomerase RNA component, with a neomycin resistance (NEO) gene.

Figure 2 is a restriction map of the genomic mTR gene.

Figure 3 shows the *Xho*I and *Xba*I fragments with introduced *Sac*I (Sc*) restriction sites.

Figure 4 shows the *Xho*I and *Xba*I genomic fragments with inserted *Sac*I* (Sc*) sites cloned into the Bluescript SK- and KS+ plasmids and into pPNT-mTRΔ.

Figure 5 is the nucleotide sequence (SEQ ID NO:1) of DNA encoding the human telomerase RNA component with the telomeric repeat template underlined and the start (ST) and end (STP) of transcription sites marked.

Figure 6 is the nucleotide sequence (SEQ ID NO:2) of DNA encoding the mouse telomerase RNA component with the telomeric repeat template underlined.

Figure 7A-7B is a comparison of DNA encoding the human (hTR), mouse (mTR), rat (rTR) (SEQ ID NO:3), hamster (cTR) (SEQ ID NO:4), and bovine (bTR) (SEQ ID NO:5) telomerase RNA components showing the conserved sequences between the molecules.

Figure 8 is the nucleotide sequence (SEQ ID NO:6) of the *Tetrahymena* 80 kD telomerase protein component gene. The nucleotide sequence is derived from genomic and cDNA clones.

Figure 9 is the amino acid sequence (SEQ ID NO:7) of the *Tetrahymena* 80 kD protein component deduced from the nucleotide sequence shown in Figure 8.

Figure 10 is the nucleotide sequence (SEQ ID NO:8) of

the *Tetrahymena* 95 kD telomerase protein component gene.

Figure 11 is the amino acid sequence (SEQ ID NO:9) of the *Tetrahymena* 95 kD protein component deduced from the nucleotide sequence shown in Figure 10.

Figure 12 is the nucleotide sequence of a genetically-engineered p80 telomerase protein component gene.

Figure 13 is the nucleotide sequence of a genetically-engineered p95 telomerase protein component gene.

Detailed Description of the Invention

This invention provides transgenic nonhuman organisms, such as transgenic mice, in which telomerase activity is altered. In one embodiment, telomerase activity is reduced or absent because one or more of the endogenous genes encoding a telomerase component is lacking or does not encode a functional telomerase component. In this example, all or a portion of the endogenous or wildtype gene has been replaced with an exogenous nucleic acid sequence, *i.e.*, a sequence not normally found in the corresponding wildtype species. A knockout organism is one in which both of the endogenous genes for a telomerase component have been completely disabled. Telomerase activity is absent in a knockout organism; *i.e.*, telomerase activity is absent in somatic and germline cells compared to a wildtype organism of the same species. The transgenic organisms described include heterozygous and homozygous organisms. Heterozygotes include transgenic organisms in which one of the two copies of a telomerase component gene is altered and telomerase activity is the same or is reduced in somatic or germline cells compared to a wildtype organism of the same species.

The invention further comprises cells or tissues of transgenic nonhuman organisms wherein telomerase expression is otherwise altered. For example, transgenic nonhuman organisms are provided in which telomerase activity is

activated through the insertion of one or more genes encoding a telomerase component. Alternatively, a DNA construct is incorporated into the genome of the organism and selectively regulates genes encoding telomerase so that telomerase activity can be induced or inhibited at particular times or in selected tissues.

Transgenic nonhuman organisms are also provided wherein the organism contains all or part of an exogenous DNA sequence encoding a telomerase component gene from another species. The resulting telomerase molecules comprise at least part of an exogenous telomerase component which replaces the corresponding nucleotide sequence of the endogenous gene.

As used herein, the term "transgenic nonhuman organism" means organisms which result from alteration of one or more endogenous genes encoding a telomerase component (founder organisms) and all subsequent generations. The term "descendants" refers to any and all future generations derived or descending from a "founder" transgenic organism, *e.g.*, an organism containing an exogenous construct as part of its genomic DNA and able to transmit this construct through its germ cells. Thus, descendants of any successive generation are included herein if the descendants contain the alteration or transgene as part of their genome.

The organisms referred to in this application include all vertebrate and invertebrate multicellular organisms described in the kingdom Animalia, unicellular and multicellular fungi, and all animal-like protists. The term "animal-like protists" includes all unicellular eukaryotes that are absorptive or ingestive heterotrophs, including, *e.g.*, *Tetrahymena sp.*, *Amoeba, sp.*, and parasitic protozoa such as *Trichomonas sp.*, *Giardia sp.*, *Entamoeba sp.*, *Plasmodium sp.* and *Leishmania sp.*. See, *e.g.*, Lee, J.J., *et al.* (eds.) *An Illustrated Guide to the*

Protozoa, Society of Protozoologists, Lawrence KS, 1985. All references to nonhuman organisms are meant to include species of Animalia, Fungi, and animal-like species of the Protista, except in instances where it is obvious that a multicellular animal is intended (*e.g.*, references to blood, vertebrate, ES cells, an organ, tumor, etc.).

Although mice homozygous for the deletion of a telomerase component (homozygous null) are known (see Example 6), cells or tissues can be derived from any stage of development of an organism homozygous for a telomerase deletion, such as a homozygous embryo, fetus, or immature animal. The cells can be cultured, using standard cell or tissue culture techniques, and then used to study the functioning of cells in the presence of various agents and can function as implants in organisms with normal telomerase activity.

Implants can be especially useful when inserted into nude mice which are prone to tumor formation. The mice, for example, can be exposed to carcinogens and the knockout tissues observed to determine if tumor formation occurs in the absence of telomerase. Therefore, the present invention provides a method to determine the relationship between telomere shortening, telomerase activity and tumor formation.

As an alternative, an exogenous gene encoding a telomerase component can be combined with an inducible promoter and integrated into the genome of a knockout organism of the present invention so that telomerase expression can be turned on or off at certain times or in particular tissues of the transgenic organism. Also, a "conditional knockout" can be generated (Example 8) if a knockout transgenic organism has a significantly reduced viability, making it difficult to breed, or if the absence of telomerase in all tissues results in additional phenotypes that complicate studies of the effects of

telomerase deletion. In one embodiment, a mouse is produced that is heterozygous for the mTR knockout while the endogenous mTR gene is flanked by a recombination site on either side of the gene. When the mouse is bred to a mouse with a gene encoding recombinase, most of the cells of the F1 mouse have one copy of the recombinase. Thus, in selected tissues, telomerase expression is completely knocked out.

The alternative transgenic nonhuman organisms described above are useful if a knockout embryo of an animal species other than a mouse cannot survive. Telomerase transcription in the knockout can be turned off at any stage of development to ascertain when and where telomerase is essential, especially in growth and aging processes, or in tumor formation. Alternatively, telomerase transcription in an adult animal can be turned on or off in selected tissues so that the effects of a drug can be determined with and without telomerase activity.

Modifications of organisms through transgenic procedures can produce telomerase alterations of various types, including insertions, deletions, substitutions, or additions of nucleic acids or amino acids, or any combination of the preceding. A specific example of such a modification is the inactivation of the telomerase RNA component gene by site-specific integration of a nucleotide sequence that replaces the endogenous gene encoding the RNA telomerase component of the mouse, as described in the Exemplification. Using this technique to knock out a gene by gene targeting avoids problems associated with the use of antisense RNA to disrupt functional expression of a gene product. As described in Examples 2 and 3, a selectable marker gene, flanked by DNA sequences isogenic to the sequences at the 5′ and 3′ most ends of the gene segment to be replaced, is inserted into the exogenous DNA construct so that homologous recombination between the exogenous

construct and the endogenous target DNA results in insertion of the selectable marker gene into a coding region or essential regulatory element of the target gene. The term "selectable marker gene" refers to a nucleic acid sequence whose expression allows for selection of targeted cells that have stably incorporated the exogenous DNA, making it possible to screen the targeted cells or derivatives of these cells for heterozygosity.

Examples of genes encoding selectable markers include, but are not limited to: genes conferring resistance to compounds such as antibiotics, genes conferring the ability to grow on selected substrates, and genes encoding proteins that produce detectable signals, such as dye staining or luminescence. A wide variety of such markers are known and available, including, for example, antibiotic resistance genes such as the neomycin resistance gene (NEO), Southern, P. and Berg, P. (1982) *J. Mol. Appl. Genet. 1*:327-341 (1982); and the hygromycin resistance gene (HYG) Kaster, K., *et al.* (1983) *Nucleic Acids Res. 11*:6895-6911, and Te Riele, H., *et al.* (1990) *Nature* 348:649-651. Other selectable markers for use in organism cells, and plasmids carrying a variety of genes encoding selectable markers, are described in Sambrook, J., *et al. Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989).

The invention also provides embryonic stem (ES) cells and embryonic stem cell lines in which an endogenous telomerase component gene has been knocked out (deleted or otherwise disabled) by the methods described herein. A further subject of the invention are ES cells and ES cell lines in which a telomerase component gene has been replaced with a different gene. The replacement gene can encode a chimeric product; for example, a gene encoding a mouse/human telomerase RNA component wherein the human template sequence replaces the mouse template sequence in a

mouse telomerase RNA component gene. Such a construct is useful to determine if human telomerase can be produced and are capable of elongating the telomeres of mouse chromosomes. A replacement gene can also encode a complete or functional portion of a telomerase RNA or protein component sequence from another organism species. Several examples of genes encoding telomerase RNA or protein components are shown in Figures 5, 6, 7A-7B, 8, and 10. Active telomerase comprising chimeric combinations of RNA and protein components of different species can provide valuable information concerning phylogenetic origins and can also provide therapeutic models for recombinant molecules that can be used in gene therapy. It could further be determined if a construct for replacement of the RNA component gene which retains the conserved nucleotides of the genes encoding telomerase RNA component of different species, as shown in Figure 7A-7B, can produce active telomerase in any organism.

Transgenic organisms in which the gene encoding telomerase can be turned on or off in cells or tissues are useful for studying aging and control of tumor growth, as well as many other growth processes. For this purpose, chimeric sequences which comprise an inducible promoter coupled to a telomerase component gene can be used to increase or decrease telomerase activity in particular cells and tissues at preferred times. For example, the tetracyline-responsive cytomegalovirus promoter effectively allows differential control of the activity of genes in mammalian cells. Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA 89*:5547-5551. Cells and tissues comprising telomerase component genes under such controls are useful in the study of cellular senescence and immortalization.

The capability to maintain chromosomal replication (i.e., cell division) has many therapeutic possibilities,

among which is gene therapy. During gene therapy, cells are normally extracted from individuals, the genome of the cell is altered to correct a genetic defect or produce a required protein (such as erythropoietin), and the altered cells returned to the individual. If immortalized cell lines are not used, the extracted cells are limited in their number of cell divisions so that small numbers of therapeutic cells are produced. Instead of using immortalized cell lines, which raises the possibility of tumor induction, blood or tissue cells of an individual can be genetically modified to incorporate genes encoding telomerase as well as nucleic acids encoding a therapeutic protein or proteins. The encoded telomerase can lengthen the number of cell divisions, thus lengthening the life span of the modified cells prior to therapy or after introduction into an individual. Larger numbers of therapeutic cells could be produced for delivery to the individual. (The individual from which the cells were obtained or another individual.) The prophylactic and therapeutic possibilities are not limited, and include any application wherein the control of cell division, is beneficial to the organism.

A replacement sequence for an endogenous telomerase gene, *e.g.*, the sequence diagrammed in Figure 1B, can encode a marker, such as a neomycin resistance gene. The marker gene can be coupled to a promoter which is constitutive or inducible. It is expected that the expression of the marker will be used to detect cells wherein the marker DNA sequence has replaced an endogenous telomerase component sequence and homologous recombination has occurred.

This invention also includes constructs, particularly DNA constructs, useful for producing the transgenic nonhuman organisms, such as the transgenic mice, described herein. The DNA comprising the knockout or replacement

construct will usually include one or more exon(s) and/or intron(s), or regions thereof of a gene encoding a telomerase component, and/or a promoter region. Any DNA that is the functional equivalent of a telomerase component can be used. The functional equivalent of a telomerase component gene is a gene encoding a molecule that is capable of combining with other endogenous or exogenous telomerase components to form an active telomerase enzyme. Other DNA can comprise promoters, sequences which encode markers, and modified or synthetic gene sequences.

Generally, the DNA construct will be at least about 1 kilobase (kb) in length and preferably 3-15 kb in length, thereby including sufficient complementary sequence for recombination when the exogenous construct is introduced into the genomic DNA of the targeted cell. Larger constructs may be required if the replacement is comprised of several genes and promoters or regulatory sequences. The preferred genes to be altered are any or all of the genes encoding the RNA component and the protein component(s) of telomerase.

There may be one or more protein components comprising the telomerase enzyme, depending on the species of cell targeted. The *Tetrahymena* telomerase ribonucleoprotein, for example, contains a 95 kb protein (p95), an 80 kb protein (p80) (Figures 9 and 11, respectively), and a 159 nucleotide RNA component. The RNA component contains a short internal sequence which serves as a template for synthesis of the G-rich strand of a telomeric repeat. The two protein components have different nucleic acid binding properties: p95 binds specifically to telomeric primer DNA, whereas p80 binds most specifically to the telomerase RNA.

Both the RNA component and the protein component of telomerase are essential for enzymatic activity. Therefore, it is only necessary to ensure that one of the

telomerase components, either the RNA component or a protein component, is not transcribed or that the product of the gene is not functional to diminish or suppress the activity of telomerase. As used herein, the term "nonfunctional" refers to the inability of the expressed component to combine with any of the other telomerase components to form an enzyme capable of adding telomeric repeats to chromosome ends or to the inability to perform its specific enzymatic role once combined.

The DNA sequence used in producing the knockout construct is digested with a particular restriction enzyme selected to cut at a location such that a new DNA sequence encoding a marker gene can be integrated in a position within this DNA sequence and transcription of the endogenous gene will be prevented after insertion of the knockout construct into the chromosome. Those of skill in the art will recognize the various factors that will affect the selection of useful restriction sites for this purpose. The marker gene to be inserted will normally have a polyA addition site attached to its 3′ end. The marker can be operably linked to its own promoter or to another strong promoter from any source that is active or can easily be activated in the cell into which it is integrated. Alternatively, the marker gene can be transcribed using the promoter of the gene that is suppressed.

By flanking the exogenous gene of the construct with sequences substantially isogenic with the target DNA in the host cell, it is possible to introduce the gene in a site-specific fashion at the targeted location. Using this approach, a gene from any source (*e.g.*, bacterial, fungal, plant, animal) can be introduced into the organism host cell to impart new telomerase characteristics to the cell, replace a telomerase component, or otherwise alter the cell to produce desired telomerase products which can then be used to assay *in vivo* for the effects of new drugs.

Isolated cells and tissues from the organism or from its descendants can also be used for assays *in vitro*.

An example of a knockout construct of the present invention is shown in Figures 1B and 4. The plasmid pPNT-mTRΔ comprises the selectable marker gene for neomycin resistance inserted in a position where it will replace the endogenous gene which encodes the RNA component of mouse telomerase. See Figure 1A. The targeted endogenous sequence to be deleted comprises a 3.9 kb segment which includes the gene encoding the mouse telomerase RNA component (mTR), and is located between a 3.3 kb *Xba*I fragment at the 5′ end and a 4.0 kb *Xho*I fragment at the 3′ end of the endogenous sequence.

The replacement sequence is ligated into the genomic DNA sequence after the genomic DNA sequence has been digested with the appropriate restriction enzymes. To make the ends of the fragments compatible for ligation, the ends can be blunted, for example by Klenow fragment, or all fragments can be cut with enzymes that generate compatible ends. Methods for carrying out these procedures are well known to those skilled in the art and can be found in Sambrook *et al.*, *supra*. The ligated construct can be inserted directly into ES cells or it can be incorporated into a suitable vector for amplification prior to insertion.

Transgenic animals from any species of rodent, including without limitation, rabbits, rats, hamsters, and mice, can be produced, as can other nonhuman transgenic organisms, such as dog, cat, pig, sheep, cow and primates. In most cases, the ES cells used to produce the transgenic animal will be of the same species as the transgenic animal to be generated. Thus, for example, mouse embryonic stem cells will usually be used for the generation of knockout mice. Transgenic animals can be prepared using methods known to skilled artisans. See, for example, Hogan, *et al.*

(eds.), *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1986).

The ES cells can be selected for their ability to integrate into and become part of the germ line of a developing embryo so as to result in germline transmission of the inserted DNA construct. Thus, any ES cell capable of such integration is suitable for use herein.

Methods of insertion of DNA vectors into ES cells include, but are not limited to, electroporation and microinjection. The insertion of DNA constructs into ES cells by electroporation is described in Example 3 of the Exemplification.

ES cells can be screened for the inserted construct by a variety of methods, which can be used alone or in combination. If a marker sequence has been incorporated, the appropriate conditions and procedures are applied to identify the marker product in the cells as an indication of the presence of the construct. For example, where the marker is an antibiotic resistance gene, such as the neomycin resistance gene, the cells are contacted with a concentration of the antibiotic (neomycin) which is lethal to cells which do not express a functional neomycin resistance gene. Those cells that survive are presumed to have integrated the construct into their genome because they are resistant to neomycin, whereas the wildtype cells are not. Or, the marker gene can encode an enzyme whose activity is detected by contacting the cells with the appropriate substrate for the encoded enzyme, then assaying for enzyme activity (or product).

Alternatively, or in addition, a Southern blot of the genomic DNA of the ES cells can be probed with a DNA sequence which hybridizes to the marker sequence. Probes such as the 1.1 kb and 1.2 kb probes shown in Figure 1A and 2, can be used to distinguish the genome of ES cells or

tissues of transgenic organisms which are either unchanged or in which the inserted construct has randomly integrated into the genome from those in which the knockout construct has integrated into targeted site (mTR) of the genome. These probes are especially useful to determine if the germline cells of the transgenic organism include a knockout construct for the telomerase RNA component.

Figure 1A illustrates a 1.1 kb probe at the 5′ flanking end of the wildtype chromosome and a 1.2 kb probe at the 3′ flanking end of the same chromosome, either of which can be used to determine if the knockout construct of pPNT-mTRΔ has replaced the endogenous mouse telomerase RNA component gene through homologous recombination. The 5′ flanking probe is located between *Sac*I (Sc) and *Xba*I; the 3′ flanking probe is located between *Xho*I and *Sac*I. Following digestion with enzyme *Sac*I, the fragment lengths of DNA isolated with these probes will be shorter after excision from the knockout construct due to the insertion of a unique *Sac*I (Sc*) restriction site engineered into the *Xba*I and *Xho*I genomic fragments which are incorporated into the knockout construct (see Example 2). As shown in Figure 1A, the wildtype gene will produce a 7.0 kb fragment including the 5′ probe and a 6.5 kb fragment with the 3′ probe. If the NEO gene has replaced the mouse telomerase gene, a 4.4 kb fragment (5′ probe) or a 5.2 kb fragment (3′ probe) will be produced. Therefore, if the 5′ most probe is used for detection, wildtype cells will produce only a 7.0 kb band; a heterozygous cell will produce 7.0 kb and 4.4 kb bands, and those cells homozygous for the knockout construct (homozygous null) will produce a 4.4 kb band only. In a similar manner, the bands detected with the 3′ most probe will be 6.5 kb (wildtype cells), 6.5 kb and 5.2 kb (heterozygous cells), or 5.2 kb (homozygous null cells). Those of skill in the art will recognize that other probes can be isolated and used with these methods to determine if

replacement of a telomerase component gene has occurred in cells and tissues of a transgenic organism.

The ES cells carrying a knockout or altered construct are introduced into embryos using known methods. For example, they can be microinjected into eggs according to known protocols, such as those described in Example 5. Other methods for production of transgenic rodents are set forth in Hogan, *et al. (eds.)*, *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1986).

The developing ES cell carrying the construct is injected into a blastocyst and implanted into a pseudopregnant female of an appropriate strain and allowed to develop into offspring. As an option, the blastocyst can be fertilized and incubated *in vitro* prior to introduction into the female.

Following implantation, gestation, and birth, the nonhuman organism which develops from the embryo is a transgenic nonhuman organism, preferably one in which the germline cells contain the altered construct. These founder organisms, which are likely to be heterozygous for the altered construct, can be bred to produce homozygotes. See Example 6. Heterozygotes, as well as being useful to generate knockout organisms, can be altered to produce further genetic modifications affecting telomerase activity resulting from the expression of an endogenous telomerase component gene. Conditional knockout organisms as described in Example 7 represent one application of a modification.

The transgenic organisms and cells derived from such organisms have a variety of uses, which depend on the telomerase component expressed and their integrated constructs. Such organisms can be used to screen for drugs or a therapeutic regimen useful for prophylactic or therapeutic treatment of diseases such as cancers and for

the regeneration of cells and tissues that do not normally divide infinitely.

More specifically, this invention provides a method of identifying an agent that is active in stimulating telomerase activity, which comprises:

a) administering an agent to a transgenic nonhuman organism, wherein the organism does not express telomerase activity or has diminished telomerase activity but has the capability to produce telomerase; and

b) assessing the effect of the agent on the telomerase activity,

wherein if the agent causes an increase in telomerase activity, the agent is active in stimulating telomerase activity.

This invention further provides a method of identifying an agent that is active in stimulating telomerase activity, which comprises:

a) administering an agent to a sample of transgenic cells or tissue, wherein the sample does not express telomerase activity or has diminished telomerase activity but is capable of producing telomerase activity; and

b) assessing the effect of the agent on the telomerase activity,

wherein if the agent causes an increase in telomerase activity, the agent is active in stimulating telomerase activity.

Additionally, this invention provides a method of identifying an agent that is active in inhibiting telomerase activity, which comprises:

a) administering an agent to a transgenic nonhuman organism, wherein the organism expresses telomerase activity; and

b) assessing the effect of the agent on the

telomerase activity,

wherein if the agent causes a decrease in telomerase activity, the agent is active in inhibiting telomerase activity.

The method can also be used with samples of transgenic cells or tissue which express telomerase and are derived from transgenic nonhuman organisms to determine if the agent inhibits telomerase activity.

Just as the organisms of this invention can be used as models to study telomerase activity, they can also provide a system in which a material suspected of being a carcinogen can be tested by exposing the organism to the material and determining neoplastic growth as an indicator of carcinogenicity. Transgenic nonhuman organisms, in particular, are very useful to develop effective therapies or regimens for combatting diseases or conditions and to ascertain the overall specific effects of a drug on telomerase activity in a living system.

The role of telomerase in the development and growth of tumors can be determined by comparing the effects of carcinogens and other tumor-inducing mechanisms in tissues wherein telomerase activity is turned on or off. See, *e.g.*, Examples 7 and 8. Telomerase activity in tumor cells maintains the severely-shortened telomeres of these cells. The absence of telomerase in tumor-forming cells could retard and even stop the growth of tumors if the dividing tumor cells lose their telomeres completely due to lack of telomerase activity.

Another important feature of the present invention is that it provides, for the first time, an organism wherein the side effects of drugs affecting telomerase activity can be ascertained and distinguished from the effects of diminished or enhanced telomerase activity. By "side effects" it is meant those effects of a drug which are not attributable to the alteration in telomerase activity.

Further, the side effects of such drugs as they affect the descendants of treated organisms can be determined. In particular, the toxicity of telomerase activity inhibitors can be tested *in vivo* using mammalian systems such as the transgenic mice described herein. For example, genes encoding human telomerase can be used to replace endogenous telomerase genes in mice, and the effects of telomerase inhibitors can be studied in a mammalian system over several generations so that any short-term or long-term side effects of the inhibitors can be documented along with the specific effects of alterations in telomerase activity. In addition, screening of these drugs for their effects on particular transgenic cells and tissues can be done *in vivo* or *in vitro*.

More specifically, this invention provides a method of identifying side effects of an agent that modifies telomerase activity in an organism, which comprises:

a) administering the agent to a transgenic nonhuman organism which is incapable of expressing telomerase; and

b) assessing the effects of the agent on the transgenic nonhuman organism,

wherein if the agent causes one or more effects on the organism, it is identified as an agent that causes one or more side effects.

In knockout organisms or tissues, shutting down the production of telomerase may result in the inability of cells to immortalize, thus, making tumor formation impossible. In addition to using knockout organisms for demonstration of these effects, cells or tissues comprising the knockout construct can be transplanted onto nude mice which have a propensity to form tumors. The absence or reduced incidenc of tumors at the transplantation sites would show that telomerase is required for tumor formation.

Alternatively, conditional knockout organisms or those

in which a telomerase component is under the control of an inducible promoter, as described *supra*, can be used to induce suppression of telomerase production either before or after tumor formation. It could then be determined if tumor formation can occur in the absence of telomerase and if suppression of telomerase causes cell division to cease in tumors.

In particular, mouse model systems for tumor formation have generated a wealth of information about cancer progression. Palmiter, R.D. and Brinster, R.L. (1985) *Cell 41*:343-345; Cory, S. and Adams, J.M. (1988) *Ann. Rev. Immunol. 6*:25-48; Hanahan, D., *et al.* (1989) *Science 246*:1265-75. The expression of oncogenes in transgenic mice provided definitive evidence that oncogenes cause tumors in mammals. Further, the finding that not all cells that express these oncogenes form tumors supported the multiple hit model for tumor progression. Brinster, R.L., *et al.* (1984) *Cell 37*:367-379; Adams, J.M., *et al.* (1985) *Nature 318*:533-538; Hanahan, D. (1985) *Nature 315*:115-122; Cory and Adams, *supra*. Mouse models for tumor suppressor genes have been developed by creating homozygous germline deletions in mice. Donehower, L.A., *et al.* (1992) *Nature 356*:215-221; Jacks, T., *et al.* (1992) *Nature 359*:295-300; Jacks, T., *et al.* (1994) *Curr. Biol. 4*:1-7; Jacks, T., *et al.* (1994) *Nature Genet. 7*:353-361. Mice that are homozygous null for a telomerase component, such as the mTR (-/-) mice, or heterozygous mTR (+/-) mice can be crossed to both oncogene expressing mice and mice deleted for tumor suppressor genes, and the mTR (-/-) oncogene-expressing offspring examined to determine if the absence of telomerase results in a lower rate of tumor formation, smaller tumor size or a lower frequency of metastasis. Further, a recombination deficient mouse can be used to generate a mTR (-/-) mouse into this background to look at the effects of recombination telomerase bypass pathways.

See Example 8.

An organism or tissue system in which telomerase activity is under the control of an inducible promoter, can also be used to study senescence. Most somatic cells lose segments of their telomeres as they divide in culture; whereas, telomeres are maintained in germline cells. Greider, C. and Blackburn, E.H. (1996) *Scientific American 274(2)*:92-97. Stimulation of telomerase activity in somatic cells of an aging transgenic nonhuman organism could answer many questions about the role of telomerase during periods of cellular senescence, especially the effects of telomerase in atherosclerosis or the decline in immunity individuals experience as they age.

Those skilled in the art will also recognize the value of the organisms, cells and tissues of this invention as a tool for understanding the growth and division of cells, and for production of cells and tissues that, in their wildtype form, are difficult to study *in vitro* because they undergo a small number of cell divisions.

EXEMPLIFICATION

Example 1

Map of mTR Genomic Locus

A 15 kb lambda genomic clone containing the mouse telomerase RNA gene was subjected to restriction enzyme digestion and various resultant fragments were subcloned into a Bluescript vector for further analysis. To map the different genomic fragments, Southern blots were probed with a 2 kb genomic fragment containing the mTR gene. Restriction enzyme sites are shown for the following enzymes; *Sac*I, *Xho*I, *Eco*RI, *Xba*I, *Eco*RV, *Bam*HI, and *Pst*I (Figure 2).

Example 2

Construction of the Targeting Vector

To generate a mTR genomic deletion construct, site-directed mutagenesis was used to introduce two new unique *Sac*I restriction sites (*Sac*I* or Sc*) into the 3.3 kb *Xba*I and 4.0 kb *Xho*I genomic fragments (Figure 1A). These unique sites were engineered so that the correct homologous recombination events in ES cells could later be identified by digestion of genomic DNA with *Sac*I. See Figures 1A and 4. Using a 1.2 kb probe (mTR probe) to the right of the mTR gene following digestion with *Sac*I yields a 6.5 kb wildtype band or a 5.2 kb band from the correctly targeted deletion allele. The probe was labeled by random primer extension using Klenow enzyme, ^{32}P-dGTP (3000 Ci/mmol), ^{32}P-dATP (3000 Ci/mmol) and cold dCTP and dTTP.

To introduce the *Sac*I* sites, both the *Xba*I and *Xho*I genomic fragments were cloned into Bluescript SK- and KS+, respectively (Figures 3 and 4), and the orientation of the clones was determined by restriction enzyme digests. Approximately 250 nt of the 3′ end of *Xba*I fragment and the 5′ end of the *Xho*I fragment were sequenced and oligonucleotides were designed to introduce the *Sac*I* sites by site-directed mutagenesis.

The engineered *Sac*I* restriction sites were introduced into a subcloned *Xba*I 4.0 kb fragment located 5′ of the transcribed region and a subcloned 3.3 kb *Xho*I fragment located 3′ of the transcribed region. The creation of these restriction sites was confirmed by sequencing and restriction enzyme digests. After the mutagenized fragments were sequenced, they were cloned into the targeting vector pPNT (Tyulewicz, V.L., *et al.* (1991) *Cell 65*:1153-1163) to generate the plasmid pPNT-mTRΔ (See Figures 1B and 4). This vector contains a Neomycin resistance gene for positive selection of resistant clones and the HSV-tk gene (Thomas, K.R. and Cappechi, M.R. (1987)

Cell 51:503-512) for counter selection in gancyclovir of incorrectly integrated constructs.

The arrangement of the two mTR genomic fragments in the targeting construct pPNT-mTRΔ is such that when homologous recombination occurs after electroporation of mouse ES cells, the mTR gene is replaced by the Neomycin resistance gene (NEO).

Example 3

Electroporation of pPNT-mTRΔ into WW6 ES Cells

WW6 ES cells (E. Ioffe, *et al.* (1995) *Proc. Natl. Acad. Sci.* 92:7357-61) were thawed and grown in ES medium with Leukemia Inhibitory Factor (LIF). The plasmid pPNT-mTRΔ was linearized with the restriction enzyme *Not*I, and the DNA was electroporated into WW6 cells by standard protocols. Ramirez-Solis, R., *et al.* (1993) *Meth. Enzymol.* 225:855-929. Cells were selected in G418 (100 μg/ml) and gancyclovir (2 μM), and positive clones were picked after 9-10 days growth into duplicate 48 well microtitre plates; one for DNA analysis, the other for both freezing at -80° C. and potential blastocyst injection. Cells in DNA plates were grown to near confluency, harvested in Proteinase K solution (0.1% SDS, 0.5 mg/ml of Proteinase K), and analyzed for homologous recombination.

Example 4

Southern Analysis of Doubly-Resistant ES Cell Clones

Genomic DNA was prepared from over 400 G418/gancyclovir resistant cells grown in one well of a 48 well-plate after digestion with Proteinase K solution for one hour at 37° C. followed by phenol extraction and isopropanol precipitation. Approximately 20 μg of DNA was digested overnight with 40 units of *Sac*I, in the presence of RNase A. After digestion, samples were loaded on a 0.8% agarose/1X TBE gel containing Ethidium bromide and

subjected to electrophoresis for 24 hr at constant voltage (30 volts). For Southern analysis, the gels were denatured and neutralized following standard protocols and transferred to a nylon filter (Hybond N+ Amersham). After transfer, filters were washed in 2X SSC and the DNA was UV-crosslinked to the membrane using a Stratalinker. Filters were pre-hybridized in high stringency solution (1% bovine serum albumin, 200 mM sodium phosphate, 15% formamide, 1 mM EDTA and 7% sodium dodecyl sulfate) at 65° C. for two hours. Hybridization with an mTR probe (described below) was carried out overnight in the same solution. After hybridization the blots were washed in 0.2X SSC and 0.1% SDS at 65° C. and exposed to autoradiographic film.

Example 5

Generation of Chimeric and Heterozygous Mice Deleted for mTR

Of the 400 Neomycin/gancyclovir resistant clones initially screened, four were found by Southern blot hybridization to contain one correctly targeted mTR locus that was deleted for the mTR coding region. These four clones were designated Tel-1, Tel-2, Tel-3 and Tel-4. Each clone was thawed from the frozen 48 well-plate and grown in culture before injection into C57BL/6J blastocysts. Three of the clones (Tel-1, Tel-2, and Tel-3) were independently injected and the blastocysts were implanted into pseudo-pregnant mice. (See Table 1 for numbers of injected mice). The chimeric mice which were born from these mothers were identified by their mosaic coat color (resulting from the agouti contribution of the ES cells and the black contribution of the blastocysts). The mice generated from the Tel-1 microinjections had over 90% ES contribution from the injected WW6 cells, based on the percent of agouti versus black coat color.

These chimeric mice were then mated to C57BL/6J to

test for germline transmission of the knocked out allele. The number and sex of chimeric and heterozygous mice from each injection are shown in Table 1. Tail DNA from the progeny of this cross was analyzed by the Southern blot procedure described above using the unique 1.2 kb genomic mTR probe. Heterozygous mTR (+/-) mice having one wild type allele and one knockout allele were considered to have germline transmission for the targeted mTR (+/-) WW6 ES cells.

Example 6

Generation of Mice Homozygous null for mTR

Homozygous knockout mice can be generated by three methods:

a) Initially a male chimeric mouse which has previously exhibited germline transmission is mated to a heterozygous female mTR knockout mouse. If telomerase null mice are viable, up to 25% of the progeny born from this cross are expected to be mTR (-/-).

b) Homozygous mTR mice are also generated by directly crossing sibling male and female heterozygotes (mTR (+/-) sibling mating). In this cross, 25% of the progeny are expected to be mTR (-/-).

c) ES cells homozygous null for mTR can be generated from intrachromosomal homologous recombination at increased concentration of G418 (higher than 1 mg/ml). These homozygous null ES cells are injected into C57BL/6J blastocysts and transferred to the uteri of pseudopregnant females. Resultant chimeric mice contain varying ES-contribution to somatic tissues. An inert molecular tag permits an assessment as to whether cells are derived from ES or host blastocysts.

The method described in b) was used to produce homozygous null mice. See Table 1.

Table 1

Production of Telomerase-Deficient Mice

Telomerase (+/-) ES Cell Clone	Number of Blastocysts injected	Number of Chimeras (%Chimerism)	Number of Germline Chimeras	Number of (+/-) Animals	Litters from (+/-) x (+/-)			
					♂	(+/+)	(+/-)	(-/-)
Tel-1	124	5♂ + 6♀ (60-95%)	4♂ + 1♀	12♂ + 12♀ DOB 9/29 -10/18/95	A	2	5	0
					B	0	3	0
					C	2	3	1
					D	1	3	0
					E	3	5	0
					F	0	3	1
					G	2	3	0
					H*	3	5	1
Tel-2	53	6♂ + 4♀ (40-80%)	mated					
Tel-3	134	9♂ + 3♀ (60-100%)	mated					
Tel-4	not injected							

* Embryos were taken prior to birth and used for generation of fibroblast cultures.

A total of 8 transfections were performed with approximately 2 x 10^7 or 4 x 10^7 WW6 ES cells per transfection. Six transfections with an average enrichment of 4.50 provided 158 clones which were analyzed by Southern blot, demonstrating one heterozygous null (+/-) cell line (Tel-1). The average enrichment for two other transfections (where 4 x 10^7 cells were electroporated), was 5.75. Three (+/-) ES clones (Tel-2, Tel-3 and Tel-4) were isolated among 280 clones screened. In summary, the homologous recombination event of telomerase occurred at 0.17% (four in 2321 clones) in WW6 ES cells. The low frequency of homologous recombination made it difficult to obtain mutant ES clones. Therefore, efficient subcloning and screening strategies were developed which permitted a more rapid and extensive analysis of candidate mutant clones. These procedures were crucial to the success of this work. Moreover, a unique ES cell line, WW6, was important to the success of the gene targeting experiments. The mixed genetic composition of WW6 provides enhanced capacity to contribute to competent germ cells. Virtually all of the mutant ES cell clones that have been generated with this cell line have passed the mutant allele through the germline.

One heterozygous null (+/-) clone (Tel-1) has transmitted through the germline, producing animals that are heterozygous null (+/-) for telomerase. Heterozygous null animals were intercrossed to examine the viability of homozygous null animals. Because WW6 ES cell line has a mixed genetic background (75% 129/sv, 20% C57Bl/6J, and 5% SJL), some of the (+/-) animals were crossed with C57Bl/6J mice to produce the inbred strain of C57Bl/6J.

To generate homozygous knockout mice, heterozygous mTR (+/-) mice were crossed and the progeny examined by Southern blotting of tail DNA. Of 46 progeny examined from

the first eight litters, three homozygous null mTR (-/-) pups were found (Table 1). The initial screening was done using *Sac*I cut genomic DNA looking for a 5.2 kb band diagnostic of the deleted allele in place of the wildtype 6.5 kb band (Figure 1A). Several control procedures confirmed that the mice were null for mTR. Genomic DNA was cut with *Eco*RI and probed with the coding region for mTR. The wildtype and heterozygous pup DNA produced a 5.0 kb band; whereas, this band was absent in the deleted mTR (-/-) DNA. PCR was also carried out, using primers in mTR and just inside the *Xho*I site to the right of the gene. A correctly-sized band of 1.3 kb was generated with the wildtype and heterozygous DNA but not with the homozygous null DNA.

Of ten pups born in the first two litters of the mTR (+/-) intercross, none were homozygous null (2 were (+/+) and 8 were (+/-)). However, the subsequent litters produced 24 homozygous null pups out of 93 born (31 homozygous normal and 62 heterozygous), indicating that animals without a gene encoding a telomerase RNA component can survive to birth. The mTR (-/-) pups are similar to their littermates in size and appearance. Both cells and tissues derived from homozygous null mice lack telomerase activity.

Example 7

Generation of Conditional mTR Deletions

Several techniques for producing conditional knockout mice are known in tissue culture (Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci.* *85*:5166-5170) and can be modified to produce mice with conditional deletions of either mTR or a telomerase protein component which are specific to certain tissues. In one procedure, the genomic region to be deleted is flanked by Lox P recombination sites. Lox P sites are DNA sequences recognized by the

bacteriophage P1 Cre recombinase. Cre-mediated recombination at Lox P sites generates a site-specific deletion which leaves only one copy of Lox P in the genome. Sauer and Henderson, *supra*. This method has been highly successful. Studies assaying the deletion of polymerase β have reported up to 95% deletion in tissues such as liver, and as much as 60% deletion, even in non-dividing tissues such as heart. Kühn, R., *et al.* (1995) *Science 269*:1427-1429. Thus a mouse can be generated wherein one allele of mTR is deleted, and the second allele is flanked by Lox P sites. Knockout mTR (-/-) tissues are then generated by the expression of Cre in specific tissues.

Although mice which ubiquitously express Cre can be generated, tissue specific expression is more useful. Several techniques can be used to produce differential expression of mTR or another telomerase component. Cre can be placed behind a tissue specific promoter to generate specific deletion in a given tissue. Gu, H., *et al.* (1994) *Science 265*:103-106. Some promoters produce low level recombination and occasional leaky expression in unrelated tissues using this technique. To avoid these problems, Cre can be placed behind a tissue specific promoter and fused to a ligand-binding domain such as the domain from the estrogen receptor. Picard, D. (1993) *Trends Cell Biol.* 3:278-280; Logie, C. and Stewart, F. (1995) *Proc. Natl. Acad. Sci. 92*:5940-5944. Fusion to ligand-binding domains results in an inactive protein until the appropriate ligand is added to release the inhibitory effect of the ligand-binding domain. Picard, D., *et al.* (1988) *Cell 54*:1073-1080. Tissue specific expression can then be induced by feeding the mice the appropriate receptor ligand. Although the estrogen receptor has been used for many studies *in vitro*, the presence of estrogen *in vivo* can activate this receptor. Thus mutant receptors can be used which respond to artificial, non-physiological drugs.

Example 8

Tumor Induction in Telomerase-deficient mice

Rates of tumor formation in four different mouse tumor progression models, RIP-Tag2 insulinomas, K14-HPV squamous cell carcinoma, Eμ-Myc lymphomas and p53 null mice can be determined. Mice which are homozygous null (-/-), wildtype (+/+) and heterozygous (+/-) for mTR are crossed to transgenic mice with high rate of tumor formation.

a) Pancreas and skin tumor models.

Telomerase expression in two mouse tumor models (RIP-Tag2 and K14-HPV16) has been extensively characterized. RIP-Tag2 mice and K14-HPV16 mice are crossed with mice heterozygous for mTR (+/-). Because genetic background affects the rate of tumor induction in mice, the mTR mice that are in a mixed background of 129 and C57B1/6J are backcrossed to the appropriate strain to produce a more homogeneous genotype prior to crossing with mouse tumor models.

For the study of RIP-Tag2 induced insulinomas, mTR (+/-) mice are crossed into the C57B1/6 strain, after which mTR (+\-) females are crossed to RIP-TAg2 males. It is not possible to introduce the RIP-TAg2 oncogene from the female side because diabetes is induced by the oncogene expression. Thus, for this model, the cross is made in only one direction. Pups from this cross are genotyped and mTR (+/-) males carrying the RIP-Tag2 construct identified. These mice are subsequently mated to mTR (+/-) females. Approximately 12.5% of the pups from this cross should be mTR (-/-) and carry RIP-Tag2. These mice can be identified through Southern blots of tail DNA. Rates of tumor formation, size of tumors and number of metastases in litter mates that are mTR (+/+), (+/-) or (-/-) are then determined. If the numbers of the mice with the appropriate genotype are low in some litters, siblings from

identical crosses can be used as controls.

Similar experiments can be carried out to examine the rate of tumor formation in the K14-HPV16 squamous cell carcinoma model mice. The mTR (+/-) mice are backcrossed to FVB/n mice (Arbeit, J.M., *et al.* (1994) *J. Virol.* *68*:4358-4368). Both male and female mTR (+/-) progeny are then crossed to K14-HPV expressing mice to generate mice carrying both mTR (+/-) and the K14-HPV transgene. Two heterozygotes carrying K14-HPV can then be crossed and Southern blots used to identify mTR (-/-) mice carrying the K14-HPV transgene.

In both the RIP-Tag2 and K14-HPV mouse models, telomerase activity appears to be present in the late stages of tumor progression, although telomerase RNA is upregulated early. Blasco, M., *et al.* (1996) *Nature Genetics* *12*:200-204. However, not all tumors are telomerase positive, suggesting that telomerase is not absolutely required for progression to late stage tumors. Tumor progression is a stochastic process, not all cells which express the oncogenes become hyperproliferative and not all hyperplasias progress to tumor formation. If telomerase is required for those tumors where it is expressed, a comparison of mTR (-/-) to mTR (+/+) mice should show a reduced frequency of tumor formation in the mTR (-/-) mice.

If telomeres in mice are sufficiently long, telomere length may not be limiting for the number of divisions required to form a tumor. There is evidence that, unlike the tissue culture model where telomeres first shorten to a critical length, telomerase induction in tumors *in vivo* may not require critical telomere shortening. Mehle, C., *et al.* (1994) *Cancer Res.* *54*:236-241. If no difference in tumor rates are found, mice with shorter telomeres can be generated and the experiment repeated using these mice.

Three different approaches can be taken to generate

mice with shortened telomeres. First, if the mTR (-/-) mice are fertile, mice with shortened telomeres can be generated in a second generation mTR (-/-) mouse. Second, mTR (+/-) mice can be examined for telomere shortening. It is possible that half of the level of mTR is not sufficient for telomere maintenance and, consequently, telomere shortening results. If this occurs, the heterozygous mTR (+/-) mice can be used as a source of mice with pre-shortened telomeres. Finally, mice can be generated from mTR (-/-) ES cells that have been grown in culture for extended periods to allow telomere shortening. mTR (-/-) ES cells are generated by re-targeting the wildtype allele in the mTR (+/-) ES cells described *supra*. A vector similar to that shown in Figure 4 can be constructed which carries a hygromycin resistance gene in place of the mTR coding regions. ES cells can be electroporated and selected for growth in hygromycin. The doubly targeted cells are identified on Southern blots as described *supra*.

b) Lymphoma induction in mTR (-/-) mice.

Telomerase activity is present in lymphocytes in both humans and mice. In human B and T cell malignancies, telomerase activity is present at higher levels than in normal cells. Counter, C.M., *et al.* (1995) *Blood* 85:2315-2320; Broccoli, D., *et al.* (1995) *Proc. Natl. Acad. Sci.* 92:9082-9086. To determine if telomerase activity is required for induction of lymphomas, lymphoma induction in Eμ-myc expressing mice (Jackson Laboratories, Bar Harbor, ME) with or without mTR can be compared. Eμ-myc mice express the c-myc gene behind an immunoglobin enhancer. These mice reproducibly develop pre-B cell lymphoma within a few months of birth. Adams, J.M., *et al.* (1985) *Nature* 318: 533-538. Crosses with mTR (+/-) mice can be used to generate Eμ-myc expressing, mTR (+/-) heterozygotes. The rapidity with which lymphomas develop in this model can

allow a tumor-suppressor phenotype to be identified in mTR (+/-) and (-/-) mice. Thus the generation of lymphomas in genetically identical litter mates that are mTR (+/+) or (+/-) can be compared. Finally, although tumors are generated at early ages in Eμ-myc expressing mice, it has been possible to generate males that will live to sexual maturity and to cross these males. Elson, A., *et al.* (1995) *Oncogene 11*:181-190. Eμ-myc male, mTR (+/-) mice can be crossed to mTR (+/-) females to generate homozygous mTR (-/-) mice expressing Eμ-myc. The onset of lymphoma development and viability of the mice can then be monitored. If telomerase is essential for the long-term survival of B cells, lymphoma development may be retarded although immune dysfunction may still occur due to the loss of B cells. The lymphoma cells from Eμ-myc mice are easily cultured *in vitro*. Schmidt, E.V., *et al.* (1988) *Proc. Natl. Acad. Sci. 85*:6047-6051; Adams, J.M., *et al.* (1985) *Nature 318*:533-538. Pre-B cells will be cultured from the lymphomas in these animals and telomere length and the *in vitro* life span of Eμ-myc expressing mTR (+/+), (+/-) or (-/-) cells can be compared.

c) Effect of p53 deletion in mTR (-/-) mice.

The p53 gene is a tumor suppressor gene which is frequently mutated in a wide variety of human cancers. Hollstein, M., *et al.* (1991) *Science 253*:49-53. Mice heterozygous for p53 develop tumors by around 16 months of age. Most of these tumors have suffered the loss of the wildtype allele of p53. Greater than 90% of mice with a complete (-/-) germline deletion of p53 develop tumors by 3-6 months of age. Donehower, L.A., *et al.* (1992) *Nature 356*:215-221; Jacks, T., *et al.* (1994) *Curr. Biol. 4*:1-7. To determine if the absence of telomerase decreases the tumor incidence or tumor size, mice doubly deficient for p53 and mTR can be generated. mTR (+/-) mice can be mated

to p53 (+/-) mice and the tumor incidence and the tumor spectrum of the progeny examined for both litter mates and siblings with the following genotypes: mTR (+/+) p53 (+/+), mTR (+/+) p53 (+/-), mTR (+/+) p53 (-/-), mTR (+/-) p53 (+/-), mTR (+/-) p53 (-/-) and mTR (-/-) p53 (-/-). Since many human tumors are p53 negative, the affect of telomerase deletion on p53 null cells has important clinical implications. p53 null cells exhibit dramatic genomic instability. See, Lane, D.P. (1992) *Nature* 358:15-16. Telomere loss is also predicted to lead to genomic instability; the combination of telomerase and p53 loss may lead to cell death. If mTR (-/-) p53 (-/-) mice have reduced tumor incidence, telomerase inhibitors could be useful for treatment of p53-tumors in humans. Currently, p53 tumors are the most resistant tumors to chemotherapy.

d) mTR deletion in recombination deficient mice.

In the tumor induction models described above, telomerase negative tumors could survive due to telomere elongation via a recombination pathway. To date, no mice have been generated with a germline deficiency in recombination. In yeast, the Rad 52 recombination pathway is the best candidate for a potential mediator or telomere recombination because this pathway is essential for telomerase bypass in yeast. Lundblad, V. and Blackburn, E.H. (1993) *Cell* 73:347-360. Preliminary evidence suggests that deletion of the mouse Rad 52 gene is lethal. However, other genes in the same pathway could also allow recombinational bypass of telomerase deletion. The mouse homologues of Rad54 and Rad 51 have been cloned and Rad 54 (-/-) ES cells have been produced that are viable and recombination deficient. If either the Rad 54 or Rad 51 (-/-) mice are viable or if the heterozygotes show reduced recombination, these animals can be crossed to mTR (+/-) mice to generate recombination deficient mTR (-/-) mice.

Fibroblasts from these cells can be transformed with Tag and ras, and tested for their ability to form tumors in nude mice as described *supra*. Ultimately, tumor induction in the transgenic models described above can be examined to determine if recombination deficiency reduces the growth or formation of potential telomerase negative tumors.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

Claims

We claim:

1. A nonhuman transgenic organism wherein at least one endogenous gene encoding a telomerase component is altered and telomerase activity is altered.

2. The organism of Claim 1 wherein the endogenous gene encodes all or part of the telomerase RNA component.

3. A cell or tissue obtained from the organism of Claim 1.

4. The organism of Claim 1 wherein the expression of telomerase activity is reduced as compared to a wildtype organism of the same species.

5. A cell or tissue of the organism of Claim 4.

6. A nonhuman transgenic organism wherein pPNT-mTRΔ is incorporated into the germline cells at the mTR locus.

7. A somatic or germline cell of a nonhuman animal comprising an exogenous telomerase gene sequence.

8. A germline cell according to Claim 7 which is an embryonic stem cell.

9. The organism of Claim 1 wherein the endogenous gene encoding a telomerase component is altered through insertion into the genome of the organism of a nucleic acid sequence comprising at least a portion of an isogenic coding sequence linked to a marker sequence.

10. The marker sequence of Claim 9 that is a neomycin resistance gene.

11. A cell or tissue of the organism of Claim 9.

12. A nucleated cell of the organism of Claim 9 wherein the cell is selected from the group consisting of embryonic stem cells, embryonic fibroblasts, germline cells, somatic cells, white blood cells, and liver cells.

13. A nonhuman transgenic organism wherein an endogenous gene encoding a telomerase component, which is normally in the corresponding nonhuman wildtype organism, is not expressed.

14. The nonhuman transgenic organism of Claim 13 which is homozygous for an exogenous DNA sequence that replaces all or a part of an endogenous gene encoding the RNA component of telomerase.

15. The nonhuman transgenic organism of Claim 13 which contains no endogenous gene sequence capable of expressing functional telomerase.

16. The organism of Claim 13 which contains only one endogenous gene sequence per cell capable of expressing functional telomerase compared to the genome of a corresponding cell of a wildtype organism of the same species.

17. A nucleic acid probe which can detect the length of a telomerase gene fragment, wherein the length of the fragment determines the capability of a telomerase gene sequence to encode a functional telomerase RNA component.

18. A DNA construct comprising DNA encoding exogenous DNA between the 3.3 kb *Xba*I fragment of the 5′ end of the mouse telomerase RNA component gene and the 4.0 kb *Xho*I fragment of the 3′ end of the mouse telomerase RNA component gene.

19. The construct of Claim 18 wherein the exogenous DNA is a marker gene sequence.

20. The construct of Claim 19 wherein the marker sequence is a neomycin resistance gene.

21. The construct of Claim 18 further comprising a promoter.

22. The construct of Claim 21 wherein the promoter is an inducible promoter.

23. The construct of Claim 22 wherein the inducible promoter is a tetracycline-responsive cytomegalovirus promoter.

24. A mouse embryonic stem cell containing the DNA construct of Claim 18.

25. A plasmid comprising pPNT-mTRΔ or its functional equivalent.

26. A mouse embryonic stem cell selected from the group consisting of Tel-1, Tel-2, Tel-3 and Tel-4.

27. The transgenic nonhuman organism of Claim 1 wherein transcription of an endogenous telomerase RNA component sequence is under the control of a promoter sequence different from the endogenous promoter sequence controlling the transcription of the endogenous gene.

28. A knockout mouse wherein the endogenous gene is replaced in part or in whole by homologous recombination between the endogenous gene and a DNA construct comprising DNA encoding exogenous DNA between the 3.3 kb *XbaI* fragment of the 5′ end of the mouse telomerase RNA component gene and the 4.0 kb *XhoI* fragment of the 3′ end of the mouse telomerase RNA component gene.

29. A method of identifying an agent that stimulates telomerase activity, the method comprising:

a) administering an agent to a transgenic nonhuman organism, wherein the organism does not express telomerase activity or has diminished telomerase activity but is capable of producing telomerase activity; and

b) assessing the effect of the agent on the telomerase activity,

wherein if the agent causes an increase in telomerase activity, the agent is active in stimulating telomerase activity.

30. A method of identifying an agent that stimulates telomerase activity, the method comprising:

a) administering an agent to a sample of transgenic

cells or tissue obtained from a transgenic nonhuman organism, wherein the sample does not express telomerase activity or has diminished telomerase activity but is capable of producing telomerase activity; and

b) assessing the effect of the agent on the telomerase activity,

wherein if the agent causes an increase in telomerase activity, the agent is active in stimulating telomerase activity.

31. A method of identifying an agent that is active in inhibiting telomerase activity, which comprises:

a) administering an agent to a transgenic nonhuman organism, wherein the organism expresses telomerase activity; and

b) assessing the effect of the agent on the telomerase activity,

wherein if the agent causes a decrease in telomerase activity, the agent is active in inhibiting telomerase activity.

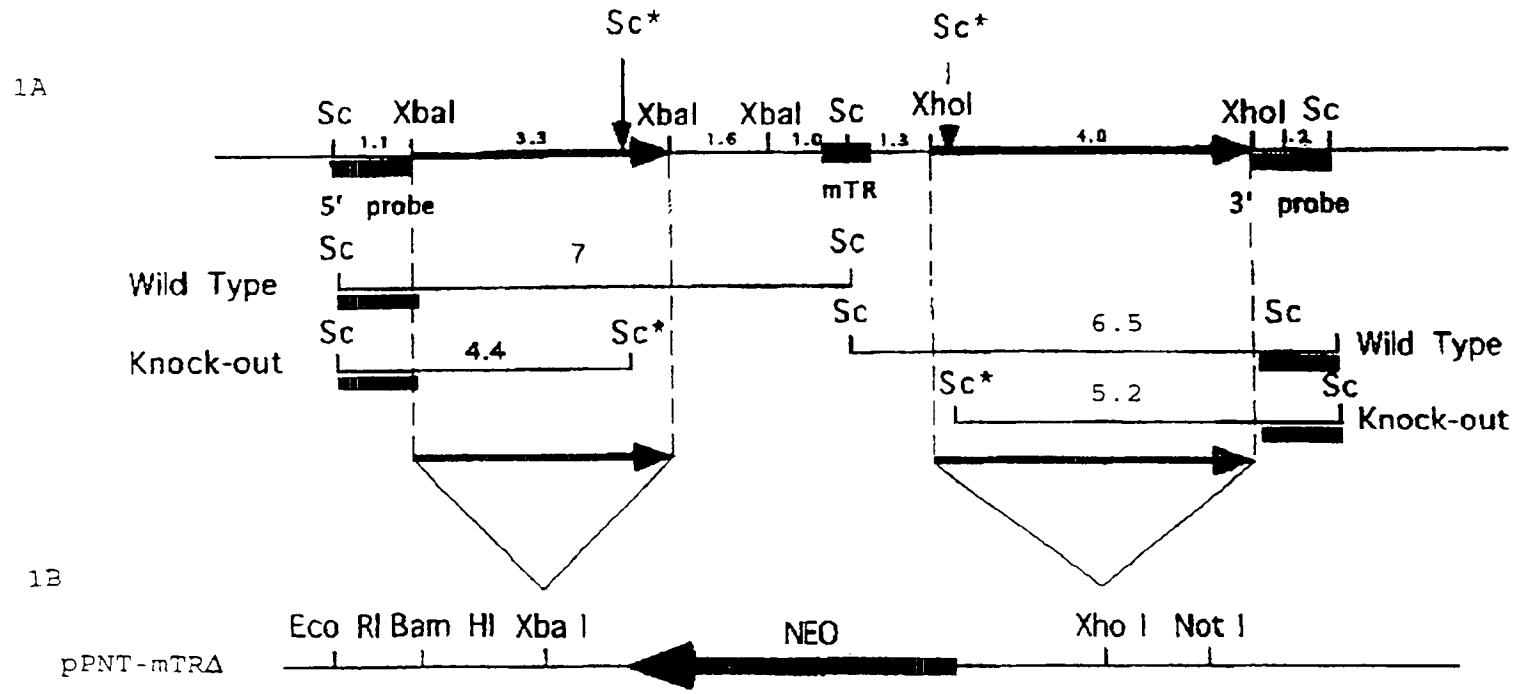
32. A method of identifying side effects of an agent that modifies telomerase activity in an organism, which comprises:

a) administering the agent to a transgenic nonhuman organism which is incapable of expressing telomerase; and

b) assessing the effects of the agent on the transgenic nonhuman organism,

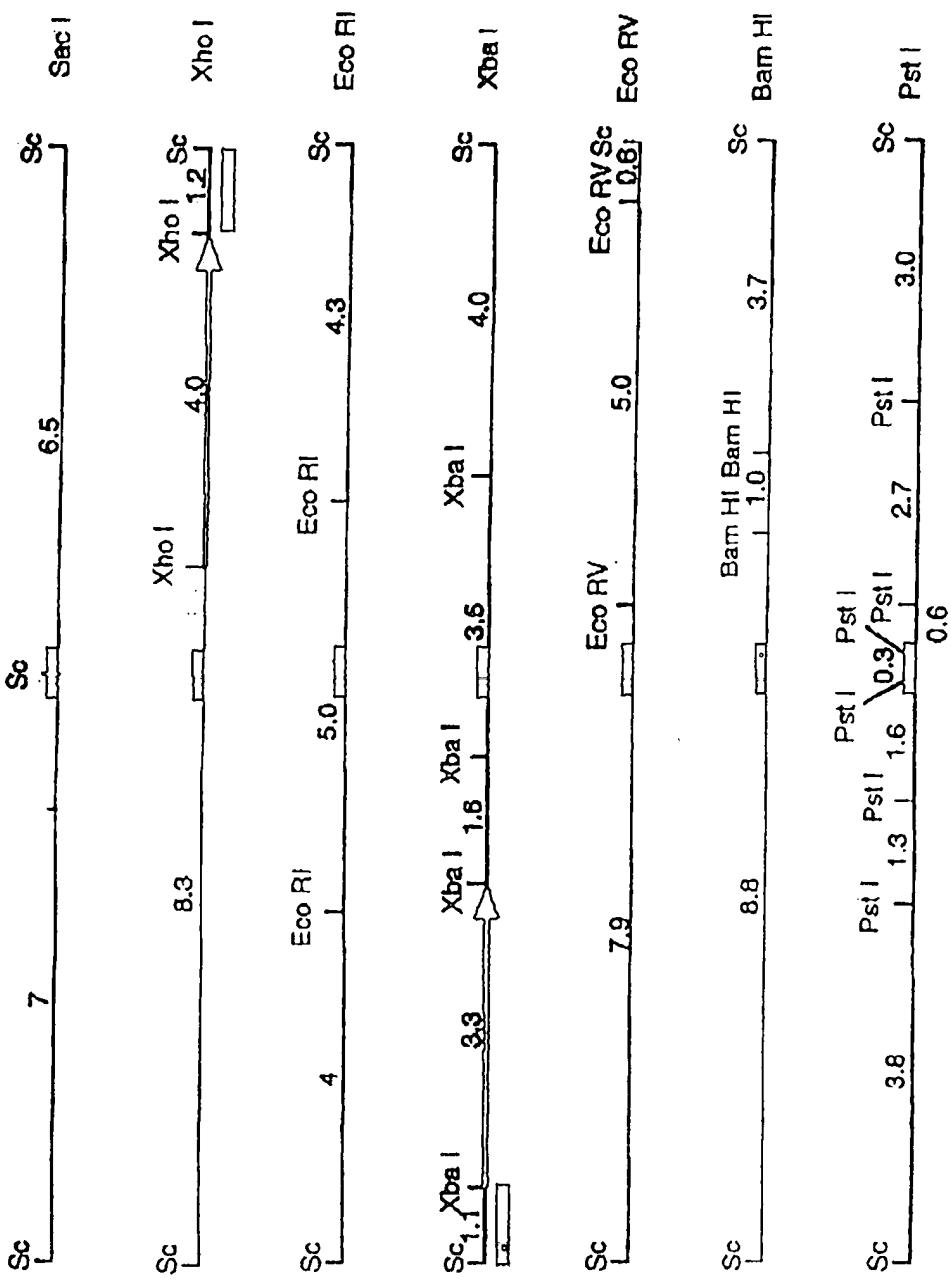
wherein if the agent causes one or more effects on the organism, it is identified as an agent that causes one or more side effects.

TARGETING CONSTRUCT FOR KNOCKING OUT MOUSE TELOMERASE RNA COMPONENT



* Site-directed mutagenesis

FIGURE 1A-1B



Restriction Map of Genomic mTR gene.

FIGURE 2

CLONING OF XBA I IN SK (-)
(determine orientation)

CLONING OF XHO I IN KS (+)
(determine orientation)

SEQUENCE 3' OF XBA I FRAGMENT
(design primer for mutagenesis)

SEQUENCE 5' OF XHO I FRAGMENT
(design primer for mutagenesis)

INTRODUCE SAC I RESTRICTION SITE
IN BOTH FRAGMENTS :

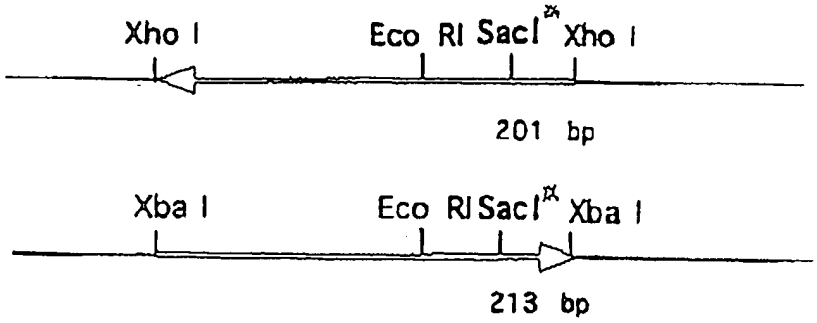


FIGURE 3

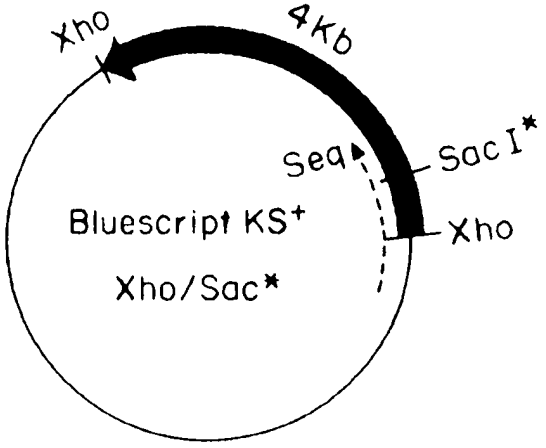


FIG. 4A

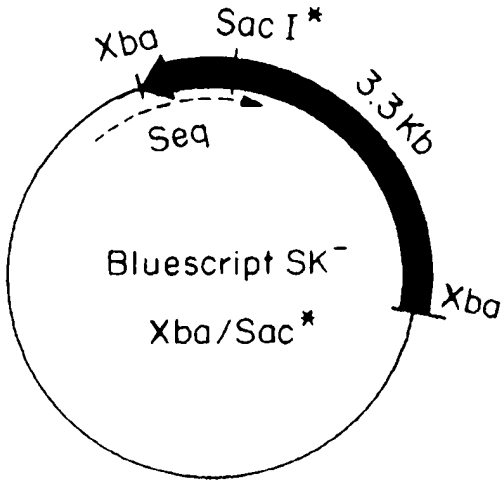


FIG. 4B

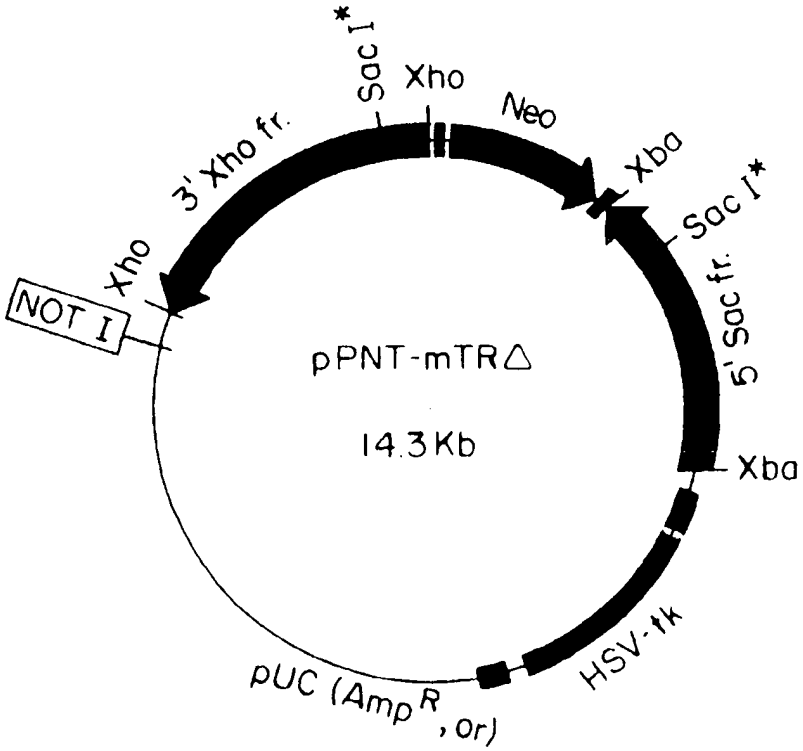


FIG. 4C

Human Telomerase RNA Component Gene

-->ST

```
GGGTTGCGGA GGGTGGGCCT GGGAGGGGTG GTGGCCATTT TTTGTCTAAC   50
CCTAACTGAG AAGGGCGTAG GCGCCGTGCT TTTGCTCCCC GCGCGCTGTT  100
TTTCTCGCTG ACTTTCAGCG GGCGGAAAAG CCTCGGCCTG CCGCCTTCCA  150
CCGTTCATTC TAGAGCAAAC AAAAAATGTC AGCTGCTGGC CCGTTCGCCT  200
CCCGGGGACC TGCGGCGGGT CGCCTGCCCA GCCCCCGAAC CCCGCCTGGA  250
GCCGCGGTCG GCCCGGGGCT TCTCCGGAGG CACCCACTGC CACCGCGAAG  300
AGTTGGGCTC TGTCAGCCGC GGGTCTCTCG GGGGCGAGGG CGAGGTTCAC  350
CGTTTCAGGC CGCAGGAAGA GGAACGGAGC GAGTCCCGCC GCGGCGCGAT  400
                                              >STP
TCCCTGAGCT GTGGGACGTG CACCCAGGAC TCGGCTCACA CATGCAGTTC  450
GCTTTCCTGT TGGTGGGGGG AACGCCGATC GTGCGCATCC GTCACCCCTC  500
GCCGGCAGTG GGGGCTTGTG AACCCCCAAA CCTGACTGAC TGGGCCAGTG  550
TGCTGCAAAT TGGCAGGAGA CGTGAAGGCA CCTCCAAAGT CGGCCAAAAT  600
GAATGGGCAG TGAGCCGGGG TTGCCTGGAG CCGTTCCTGC GTGGGTTCTC  650
CCGTCTTCCG CTTTTTGTTG CCTTTTATGG TTGTATTACA ACTTAGTTCC  700
TGCTCTGCAG ATTTTGTTGA GGTTTTTGCT TCTCCCAAGG TAGATCTCGA  750
CCAGTCCCTC AACGGGGTGT GGGGAGAACA GTCATTTTTT TTTGAGAGAT  800
CATTTAACAT TTAATGAATA TTTAATTAGA AGATCTAAAT GAACATTGGA  850
AATTGTGTTC CTTTAATGGT CATCGGTTTA TGCCAGAGGT TAGAAGTTTC  900
TTTTTTGAAA AATTAGACCT TGGCGATGAC CTTGAGCAGT AGGATATAAC  950
CCCCACAAGC TT                                           962
```

FIGURE 5

Mouse Telomerase RNA Component Gene

```
CTCGACCAAT CAGCGCGCGC CATGGGGTAT TTAAGGTCGA GGGCGGCTAG    50
GCCTCGGCAC CTAACCCTGA TTTTCATTAG CTGTGGGTTC TGGTCTTTTG   100
TTCTCCGCCC GCTGTTTTTC TCGCTGACTT CCAGCGGGCC AGGAAAGTCC   150
AGACCTGCAG CGGGCCACCC GGCGTTCCCG AGCCTCAAAA ACAAACGTCA   200
GCGCAGGAGC TCCAGGTTCG CCGGGAGCTC CGCGGCGCCG GGCCGCCCAG   250
TCCCGTACCC GCCTACAGGC CGCGGCCGGC CTGGGGTCTT AGGACTCCGC   300
TGCCGCCGCG AAGAGCTCCG CCTCTGTCAG CCGCGGGCGC GCGGGGGCTG   350
GGGCCAGGCC GGGCGAGCGC CGCGAGGACA GGAATGGAAC TGGTCCCCGT   400
GTTCGGTGTC TTACCTGAGC TGTGGGAAGT GCACCCGGAA CTCGGTTCTC   450
ACAACCCCCA TTCCCGCTGG GGAAATGCCC CGCTGCAGGG CGGGCCGCTA   500
GAACCTGCGA CTCTGGGGAA AGGGGCTTCG GTGTGAGACG GTAGCCAGCC   550
AAAGGGTATA                                               560
```

FIGURE 6

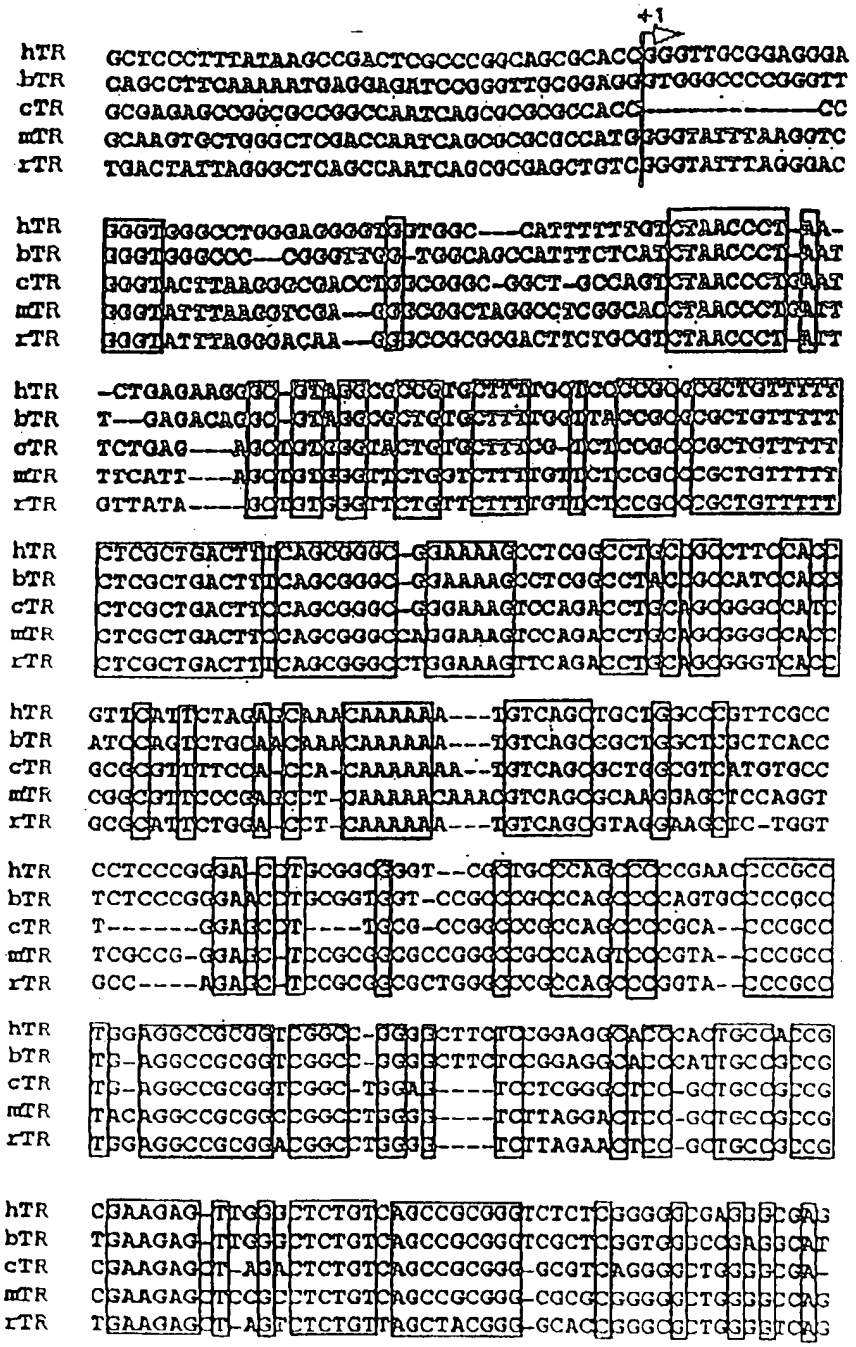

+1
hTR GCTCCCTTTATAAGCCGACTCGCCCGGCAGCGCACCGGGTTGCGGAGGGA
bTR CAGCCTTCAAAAATGAGGAGATCCGGGTTGCGGAGGGTGGGCCCCGGGTT
cTR GCGAGAGCCGGCGCCGGCCAATCAGCGCGCGCCACC----------CC
mTR GCAAGTGCTGGGCTCGACCAATCAGCGCGCGCCATGGGGTATTTAAGGTC
rTR TGACTATTAGGGCTCAGCCAATCAGCGCGAGCTGTCGGGTATTTAGGGAC
hTR GGGTGGGCCTGGGAGGGGTGGTGGC---CATTTTTTGTCTAACCCT-AA-
bTR GGGTGGGCCC--CGGGTTGG-TGGCAGCCATTTCTCATCTAACCCT-AAT
cTR GGGTACTTAAGGCCGACCTGGCGGGC-GGCT-GCCAGTCTAACCCTCAAT
mTR GGGTATTTAAGGTCGA--GGGCGGCTAGGCCTCGGCACCTAACCCTCAAT
rTR GGGTATTTAGGGACAA--GGGCCGCGCGACTTCTGCGTCTAACCCT-ATT
hTR -CTGAGAAGGGC-GTAGGCGCCGTGCTTTTGCTCCCCGCGCGCTGTTTTT
bTR T--GAGACAGGC-GTAGGCGCTGTGCTTTTGCTTACCGCGCGCTGTTTTT
cTR TCTGAG---AGCTGTGGGCTACTGTGCTTACG-TCTCCGCCCGCTGTTTTT
mTR TTCATT---AGCTGTGGGCTCTGGTCTTTTGTTCTCCGCCCGCTGTTTTT
rTR GTTATA---GCTGTGGGTTCTGTTCTTTTGTTCTCCGCCCGCTGTTTTT
hTR CTCGCTGACTTTCAGCGGGGC-GGAAAAGCCTCGGCCTGCCGGCTTCCACC
bTR CTCGCTGACTTTCAGCGGGC-GGAAAAGCCTCGGCCTACCGGCATCCACC
cTR CTCGCTGACTTCCAGCGGGC-GGGAAAGTCCAGACCTGCCAGCGGGCCATC
mTR CTCGCTGACTTCCAGCGGGCCAGGAAAGTCCAGACCTGCCAGCGGGCCACC
rTR CTCGCTGACTTTCAGCGGGCCTGGAAAGTTCAGACCTGCCAGCGGGTCACC
hTR GTTCATTCTAGAGCAAACAAAAAA---TGTCAGCTGCTGGCCCGTTCGCC
bTR ATCCAGTCTGCAACAAACAAAAAA---TGTCAGCCGCTGGCTCGCTCACC
cTR GCGCGTTTTCCA-CCA-CAAAAAAA--TGTCAGCGCTGGCGTCATGTGCC
mTR CGGCGTTCCCGAGCCT-CAAAAACAAACGTCAGCGCAAGGAGCTCCAGGT
rTR GCGCATTCTGGA-CCT-CAAAAAA---TGTCAGCGTAGGAAGCTC-TGGT
hTR CCTCCCGGGAA-CGTGCGGCGGGT--CGCTGCCCAGCCCCCGAACCCCGCC
bTR TCTCCCGGGAACCTGCGGTGGT-CCGCCCGCCCAGCCCCAGTGCCCCGCC
cTR T-------GGAGCCT----TGCG-CCGGCCCGCCAGCCCCGCA--CCCGCC
mTR TCGCCG-GGAGC-TCCGCGGCGCCGGCCCGCCAGTCCCGTA--CCCGCC
rTR GCC-----AGAGC-TCCGCGGCGCTGGCCCCGCCAGCCCGGTA--CCCGCC
hTR TGGAGGCCGCGGTCGGCC-GGGGCTTCTCCGGAGGCACCCACGTGCCACCG
bTR TG-AGGCCGCGGTCGGCC-GGGGCTTCTCCGGAGGCACCCATTGCCGCCG
cTR TG-AGGCCGCGGTCGGC-TGGAG----TCCTCGGCCTCC-GCTGCCGCCG
mTR TACAGGCCGCGGCCGGCCTGGGG----TCTTAGGACTCC-GCTGCCGCCG
rTR TGGAGGCCGCGGACGGCCTGGGG----TCTTAGAACTCC-GCTGCCGCCG
hTR CGAAGAG-TTGGGCTCTGTCAGCCGCGGGTCTCTCCGGGGCCGAGGGCGAG
bTR TGAAGAG-TTGGGCTCTGTCAGCCGCGGGTCGCTCGGTGGGCCGAGGCAT
cTR CGAAGAGCT-AGACTCTGTCAGCCGCGGG-GCGTCAGGGCCTGGGGCGA-
mTR CGAAGAGCTCCGCCTCTGTCAGCCGCGGG-CGCGCGGGGCCTGGGGCCAG
rTR TGAAGAGCT-AGTCTCTGTTAGCTACGGG-GCACCGGGCGCTGGGGTCAG

FIGURE 7A

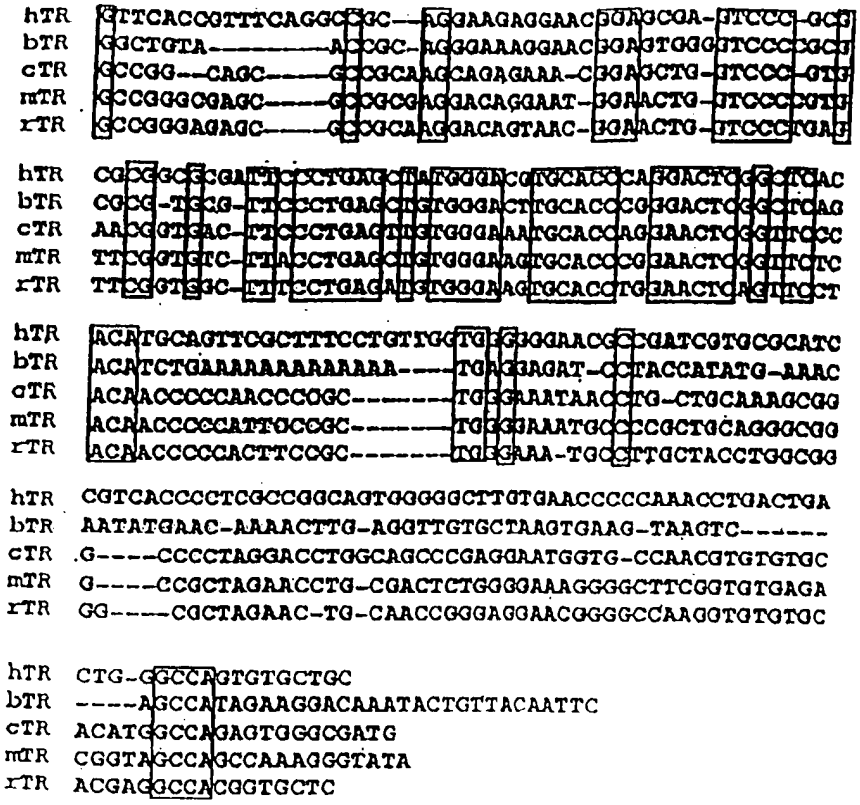


FIGURE 7B

Tetrahymena 80kD Telomerase Protein Component Gene

```
   1 aactcattta attactaatt taatcaacaa gattgataaa aagcagtaaa taaaacccaa
  61 tagatttaat ttagaaagta tcaattgaaa aatggaaatt gaaaacaact aagcacaata
 121 gccaaaagcc gaaaaattgt ggtgggaact tgaattagag atgcaagaaa accaaaatga
 181 tatataagtt agggttaaga ttgacgatcc taagcaatat ctcgtgaacg tcactgcagc
 241 atgtttgttg taggaaggta gttactacta agataaagat gaaagaagat atatcatcac
 301 taaagcactt cttgaggtgg ctgagtctga tcctgagttc atctgctagt tggcagtcta
 361 catccgtaat gaactttaca tcagaactac cactaactac attgtagcat tttgtgttgt
 421 ccacaagaat actcaaccat tcatcgaaaa gtacttcaac aaagcagtac ttttgcctaa
 481 tgacttactg gaagtctgtg aatttgcata ggttctctat atttttgatg caactgaatt
 541 caaaaatttg tatcttgata ggataacttc ataagatatt cgtaaggaac tcactttccg
 601 taagtgttta caaagatgcg tcagaagcaa gttttctgaa ttcaacgaat actaacttgg
 661 taagtattgc actgaatcct aacgtaagaa aacaatgttc cgttacctct cagttaccaa
 721 caagtaaaag tgggattaaa ctaagaagaa gagaaaagag aatctcttaa ccaaacttta
 781 ggcaataaag gaatctgaag ataagtccaa gagagaaact ggagacataa tgaacgttga
 841 agatgcaatc aaggctttaa aaccagcagt tatgaagaaa atagccaaga gatagaatgc
 901 catgaagaaa cacatgaagg cacctaaaat tcctaactct accttggaat caaagtactt
 961 gaccttcaag gatctcatta agttctgcca tatttctgag cctaaagaaa gagtctataa
1021 gatccttggt aaaaaatacc ctaagaccga agaggaatac aaagcagcct ttggtgattc
1081 tgcatctgca ccattcaatc ctgaattggc tggaaagcgt atgaagattg aaatctctaa
1141 aacatgggaa aatgaactca gtgcaaaagg caacactgct gaggtttggg ataatttaat
1201 ttcaagcaat taactcccat atatggccat gttacgtaac ttgtctaaca tcttaaaagc
1261 cggtgtttca gatactacac actctattgt gatcaacaag atttgtgagc ccaaggccgt
1321 tgagaactcc aagatgttcc ctcttcaatt ctttagtgcc attgaagctg ttaatgaagc
1381 agttactaag ggattcaagg ccaagaagag agaaaatatg aatcttaaag gtcaaatcga
1441 agcagtaaag gaagttgttg aaaaaaccga tgaagagaag aaagatatgg agttggagta
1501 aaccgaagaa ggagaatttg ttaaagtcaa cgaaggaatt ggcaagcaat acattaactc
1561 cattgaactt gcaatcaaga tagcagttaa caagaattta gatgaaatca aaggacacac
1621 tgcaatcttc tctgatgttt ctggttctat gagtacctca atgtcaggtg gagccaagaa
1681 gtatggttcc gttcgtactt gtctcgagtg tgcattagtc cttggtttga tggtaaaata
1741 acgttgtgaa aagtcctcat tctacatctt cagttcacct agttctcaat gcaataagtg
1801 ttacttagaa gttgatctcc ctggagacga actccgtcct tctatgtaaa aacttttgca
1861 agagaaagga aaacttggtg gtggtactga tttcccctat gagtgcattg atgaatggac
1921 aaagaataaa actcacgtag acaatatcgt tattttgtct gatatgatga ttgcagaagg
1981 atattcagat atcaatgtta gaggcagttc cattgttaac agcatcaaaa agtacaagga
2041 tgaagtaaat cctaacatta aaatctttgc agttgactta gaaggttacg gaaagtgcct
2101 taatctaggt gatgagttca atgaaaacaa ctacatcaag atattcggta tgagcgattc
2161 aatcttaaag ttcatttcag ccaagcaagg aggagcaaat atggtcgaag ttatcaaaaa
2221 ctttgcccct caaaaaatag gacaaaagtg agtttcttga gattcttcta taacaaaaat
2281 ctcaccccac ttttttgttt tattgcatag ccattatgaa atttaaatta ttatctattt
2341 atttaagtta cttacatagt ttatgtatcg cagtctatta gcctattcaa atgattctgc
2401 aaagaacaaa aaagattaaa a
```

FIGURE 8

Amino Acid Sequence of the *Tetrahymena* 80 kD Telomerase Protein Component

MEIENNQAQQPKAEKLWWELELEMQENQNDIQVRVKIDDPKQYL
VNVTAACLLQEGSYYQDKDERRYIITKALLEVAESDPEFICQLAVYIRNELYIRTTTN
YIVAFCVVHKNTQPFIEKYFNKAVLLPNDLLEVCEFAQVLYIFDATEFKNLYLDRILS
QDIRKELTFRKCLQRCVRSKFSEFNEYQLGKYCTESQRKKTMFRYLSVTNKQKWDQTK
KKRKENLLTKLQAIKESEDKSKRETGDIMNVEDAIKALKPAVMKKIAKRQNAMKKHMK
APKIPNSTLESKYLTFKDLIKFCHISEPKERVYKILGKKYPKTEEEYKAAFGDSASAP
FNPELAGKRMKIEISKTWENELSAKGNTAEVWDNLISSNQLPYMAMLRNLSNILKAGV
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AVKEVVEKTDEEKKDMELEQTEEGEFVKVNEGIGKQYINSIELAIKIAVNKNLDEIKG
HTAIFSDVSGSMSTSMSGGAKKYGSVRTCLECALVLGLMVKQRCEKSSFYIFSSPSSQ
CNKCYLEVDLPGDELRPSMQKLLQEKGKLGGGTDFPYECIDEWTKNKTHVDNIVILSD
MMIAEGYSDINVRGSSIVNSIKKYKDEVNPNIKIFAVDLEGYGKCLNLGDEFNENNYI
KIFGMSDSILKFISAKQGGANMVEVIKNFALQKIGQK"

FIGURE 9

Tetrahymena 95 kD Telomerase Protein Component Gene

```
   1  tcaatactat taattaataa ataaaaaaaa gcaaactaca aagaaaatgt caaggcgtaa
  61  ctaaaaaaag ccataggctc ctataggcaa tgaaacaaat cttgattttg tattacaaaa
 121  tctagaagtt tacaaaagcc agattgagca ttataagacc tagtagtaat agatcaaaga
 181  ggaggatctc aagcttttaa agttcaaaaa ttaagattag gatggaaact ctggcaacga
 241  tgatgatgat gaagaaaaca actcaaataa ataataagaa ttattaagga gagtcaatta
 301  gattaagtag caagtttaat tgataaaaaa agttggttct aaggtagaga aagatttgaa
 361  tttgaacgaa gatgaaaaca aaaagaatgg actttctgaa tagcaagtga aagaagagta
 421  attaagaacg attactgaag aataggttaa gtattaaaat ttagtattta acatggacta
 481  ccagttagat ttaaatgaga gtggtggcca tagaagacac agaagagaaa cagattatga
 541  tactgaaaaa tggtttgaaa tatctcatga ccaaaaaaat tatgtatcaa tttacgccaa
 601  ctaaaagaca tcatattgtt ggtggcttaa agattatttt aataaaaaca attatgatca
 661  tcttaatgta agcattaaca gactagaaac tgaagccgaa ttctatgcct ttgatgattt
 721  ttcacaaaca atcaaactta ctaataattc ttactagact gttaacatag acgttaattt
 781  tgataataat ctctgtatac tcgcattgct tagatttta ttatcactag aaagattcaa
 841  tattttgaat ataagatctt cttatacaag aaattaatat aattttgaga aaattggtga
 901  gctacttgaa actatcttcg cagttgtctt ttctcatcgc cacttacaag gcattcattt
 961  acaagttcct tgcgaagcgt tctaatattt agttaactcc tcatcataaa ttagcgttaa
1021  agatagctaa ttataggtat actctttctc tacagactta aaattagttg acactaacaa
1081  agtccaagat tattttaagt tcttataaga attccctcgt ttgactcatg taagctagta
1141  ggctatccca gttagtgcta ctaacgctgt agagaacctc aatgttttac ttaaaaaggt
1201  caagcatgct aatcttaatt tagtttctat ccctacctaa ttcaattttg atttctactt
1261  tgttaattta taacatttga aattagagtt tggattagaa ccaaatattt tgacaaaaca
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1381  aaacttttac acctacgttg cttaagaaac ctccagaaaa cagatattaa aacaagctac
1441  aacaatcaaa aatctcaaaa acaataaaaa tcaagaagaa actcctgaaa ctaaagatga
1501  aactccaagc gaaagcacaa gtggtatgaa atttttgat catctttctg aattaaccga
1561  gcttgaagat ttcagcgtta acttgtaagc tacccaagaa atttatgata gcttgcacaa
1621  acttttgatt agatcaacaa atttaaagaa gttcaaatta agttacaaat atgaaatgga
1681  aaagagtaaa atggatacat tcatagatct taagaatatt tatgaaacct taaacaatct
1741  taaaagatgc tctgttaata tatcaaatcc tcatggaaac atttcttatg aactgacaaa
1801  taaagattct acttttata aatttaagct gaccttaaac taagaattat aacacgctaa
1861  gtatactttt aagtagaacg aattttaatt taataacgtt aaaagtgcaa aaattgaatc
1921  ttcctcatta gaaagcttag aagatattga tagtctttgc aaatctattg cttcttgtaa
1981  aaatttacaa aatgttaata ttatcgccag tttgctctat cccaacaata tttagaaaaa
2041  tcctttcaat aagcccaatc ttctattttt caagcaattt gaataattga aaaatttgga
2101  aaatgtatct atcaactgta ttcttgatca gcatatactt aattctattt cagaattctt
2161  agaaaagaat aaaaaaataa aagcattcat tttgaaaaga tattatttat tacaatatta
2221  tcttgattat actaaattat ttaaaacact tcaatagtta cctgaattaa attaagttta
2281  cattaattag caattagaag aattgactgt gagtgaagta cataagtaag tatgggaaaa
2341  ccacaagcaa aaagctttct atgaaccatt atgtgagttt atcaaagaat catcctaaac
2401  cctttagcta atagatttg accaaaacac tgtaagtgat gactctatta aaaagatttt
2461  agaatctata tctgagtcta agtatcatca ttatttgaga ttgaacccta gttaatctag
2521  cagtttaatt aaatctgaaa acgaagaaat ttaagaactt ctcaaagctt gcgacgaaaa
2581  aggtgtttta gtaaaagcat actataaatt ccctctatgt ttaccaactg gtacttatta
2641  cgattacaat tcagatagat ggtgattaat taaatattag tttaaataaa tattaaatat
2701  tgaatatttc tttgcttatt atttgaataa tacatacaat agtcattttt agtgttttga
2761  atatatttta gttatttaat tcattatttt aagtaaataa ttatttttca atcatttttt
2821  aaaaaatcg
```

FIGURE 10

Amino Acid Sequence of the *Tetrahymena* 95 kD Telomerase Protein Component

MSRRNQKKPQAPIGNETNLDFVLQNLEVYKSQIEHYKTQQQQIK
EEDLKLLKFKNQDQDGNSGNDDDDEENNSNKQQELLRRVNQIKQQVQLIKKVGSKVEK
DLNLNEDENKKNGLSEQQVKEEQLRTITEEQVKYQNLVFNMDYQLDLNESGGHRRHRR
ETDYDTEKWFEISHDQKNYVSIYANQKTSYCWWLKDYFNKNNYDHLNVSINRLETEAE
FYAFDDFSQTIKLTNNSYQTVNIDVNFDNNLCILALLRFLLSLERFNILNIRSSYTRN
QYNFEKIGELLETIFAVVFSHRHLQGIHLQVPCEAFQYLVNSSSQISVKDSQLQVYSF
STDLKLVDTNKVQDYFKFLQEFPRLTHVSQQAIPVSATNAVENLNVLLKKVKHANLNL
VSIPTQFNFDFYFVNLQHLKLEFGLEPNILTKQKLENLLLSIKQSKNLKFLRLNFYTY
VAQETSRKQILKQATTIKNLKNNKNQEETPETKDETPSESTSGMKFFDHLSELTELED
FSVNLQATQEIYDSLHKLLIRSTNLKKFKLSYKYEMEKSKMDTFIDLKNIYETLNNLK
RCSVNISNPHGNISYELTNKDSTFYKFKLTLNQELQHAKYTFKQNEFQFNNVKSAKIE
SSSLESLEDIDSLCKSIASCKNLQNVNIIASLLYPNNIQKNPFNKPNLLFFKQFEQLK
NLENVSINCILDQHILNSISEFLEKNKKIKAFILKRYYLLQYYLDYTKLFKTLQQLPE
LNQVYINQQLEELTVSEVHKQVWENHKQKAFYEPLCEFIKESSQTLQLIDFDQNTVSD
DSIKKILESISESKYHHYLRLNPSQSSSLIKSENEEIQELLKACDEKGVLVKAYYKFP
LCLPTGTYYDYNSDRW

FIGURE 11

GGGCGGATCCATGGAGATCGAGAACAAGCAAGCTCAACAACGGAAGGCTGAGAAGCTGTGG
TGGGAGCTGGAGCTGGAGATGCAAGAGAACCAAAACGACATCCAAGTTCGTGTTAAGATCG
ACGACCCGAAGCAATACCTGGTTAACGTTACCGCTGCTTGTCTGCTGCAAGAGGGCAGCTA
CTACCAAGACAAGGACGAGGGTCGTTACATCATCACCAAGGCTCTGCTGGAGGTTGCTGAG
AGCGACCCGGAGTTCATCTGTCAACTGGCTGTTTACATCCGTAACGAGCTGTACATCCGTA
CCACCACCAACTACATCGTTGCTTTCTGTGTTGTTCACAAGAACACCCAACCGTTCATCGA
GAAGTACTTCAACAAGGCTGTTCTGCTGCCGAACGACCTGCTGGAGGTTTGTGAGTTCGCT
CAAGTTCTGTACATCTTCGACGCTACCGAGTTCAAGAACCTGTACCTGGACCGTATCCTGA
GCCAAGATATCCGTAAGGAGCTGACCTTCCGTAAGTGTCTGCAACGTTGTGTTCGTAGCAA
GTTCAGCGAGTTCAACGAGTACCAACTGGGCAAGTACTGTACCGAGAGCCAACGTAAGAAG
ACCATGTTCCGTTACCTGAGCGTTACCAACAAGCAAAAGTGGGACCAAACCAAGAAGAAGC
GTAAGGAGAACCTGCTGACCAAGCTGCAAGCTATCAAGGAGAGCGAGGACAAGAGCAAGCG
TGAGACCGGCGACATCATGAACGTTGAGGACGCTATCAAGGCTGTGAAGCCGGCCGTTATG
AAGAAGATCGCTAAGCGTCAAAACGCTATGAAGAAGCACATGAAGGCTCCGAAGATCCCGA
ACAGCACCCTGGAGAGCAAGTACCTGACCTTCAAGGACCTGATCAAGTTCTGTCACATCAG
CGAGCCGAAGGAACGTGTTTACAAGATCCTGGGCAAGAAGTACCCGAAGACCGAGGAGGAG
TACAAGGCTGCTTTCGGCGACAGCGCTAGCGCTCCGTTCAACCCGGAGCTGGCTGGCAAGC
GTATGAAGATCGAGATCAGCAAGACCTGGGAGAACGAGCTGAGCGCTAAGGGCAACACCGC
TGAGGTTTGGGACAACCTGATCAGCAGCAACCAACTGCCGTACATGGCCATGCTGCGTAAC
CTGAGCAACATCCTGAAGGCTGGCGTTAGCGACACCACCCACAGCATCGTTATCAACAAGA
TCTGTGAGCCGAAGGCTGTTGAGAACAGCAAGATGTTCCCGCTGCAATTCTTCAGCGCTAT
CGAGGCTGTTAACGAGGCGGTTACCAAGGGCTTCAAGGCTAAGAAGCGTGAGAACATGAAC
CTGAAGGGCCAAATCGAGGCTGTTAAGGAGGTTGTTGAGAAGACCGACGAGGAGAAGAAGG
ACATGGAGCTGGAGCAAACCGAGGAGGGCGAATTCGTTAAGGTTAACGAGGGCATCGGCAA
GCAATACATCAACAGCATCGAGCTGGCTATCAAGATCGCTGTGAACAAGAACCTGGACGAG
ATCAAGGGCCACACCGCTATCTTCAGCGACGTCAGCGGCAGCATGAGCACCAGCATGAGCG
GCGGCGCTAAGAAGTACGGCAGCGTTCGTACCTGTCTGGAGTGTGCTCTGGTTCTGGGCCT
GATGGTTAAGCAACGTTGTGAGAAGAGCAGCTTCTACATCTTCAGCAGCCCGAGCAGCCAA
TGTAACAAGTGTTACCTGGAGGTTGACCTGCCGGGCGACGAGCTGCGTCCGAGCATGCAAA
AGCTGCTGCAAGAGAAGGGCAAGCTGGGCGGCGGCACCGACTTCCCGTACGAGTGTATCGA
TGAGTGGACCAAGAACAAGACCCACGTTGACAACATCGTTATCCTGAGCGACATGATGATC
GCTGAGGGCTACAGCGACATCAACGTTCGTGGCAGCAGCATCGTTAACAGCATCAAGAAGT
ACAAGGACGAGGTTAACCCGAACATCAAAATCTTCGCTGTTGACCTGGAGGGCTACGGCAA
GTGTCTGAACCTGGGCGACGAGTTCAACGAGAACAACTACATCAAAATCTTCGGCATGAGC
GACAGCATCCTGAAGTTCATCAGCGCTAAGCAAGGCGGCGCTAACATGGTGGAGGTGATCA
AGAACTTCGCTCTGCAAAAGATCGGCCAAAAGTGACTGCAGACTAGTCTAGAAAGCTTGGT
ACCGCCC

FIGURE 12

GGGGCCATGGATGAGCCGTCGTAACCAAAAGAAGCCGCAAGCTCCGATCGGCAACGAGACC
AACCTGGACTTCGTTCTGCAAAACCTGGAGGTTTACAAGAGCCAAATCGAGCACTACAAGA
CCCAACAACAACAAATCAAGGAGGAGGACCTGAAGCTGCTGAAGTTCAAGAACCAAGACCA
AGACGGCAACAGCGGCAACGACGACGACGACGAGGAGAACAACAGCAACAAGCAACAAGAG
CTGCTGCCGTCGTGTTAACCAAATCAAGCAACAAGTTCAACTGATCAAGAAGGTTGGCAGCA
AGGTTGAGAAGGACCTGAACCTGAACGAGGACGAGAACAAGAAGAACGGCCTGAGCGAGCA
ACAAGTTAAGGAGGAGCAACTGCGTACCATCACCGAGGAGCAAGTTAAGTACCAAAACCTG
GTTTTCAACATGGACTACCAACTGGACCTGAACGAGAGCGGCGGCCACCGTCGTCACCGTC
GCGAGACCGACTACGACACCGAGAAGTGGTTCGAGATCAGCCACGACCAAAAGAACTACGT
TAGCATCTACGCTAACCAAAAGACCAGCTACTGTTGGTGGCTGAAGGACTACTTCAACAAG
AACAACTACGACCACCTGAACGTTAGCATCAACCGTCTGGAGACCGAGGCTGAGTTCTACG
CTTTCGACGACTTCAGCCAAACCATCAAGCTGACCAACAACAGCTACCAAACCGTTAACAT
CGACGTCAACTTCGACAACAACCTGTGTATCCTGGCTCTGCTGCGTTTCCTGCTGAGCCTG
GAGCGTTTCAACATCCTGAACATCCGTAGCAGCTACACCCGTAACCAATACAACTTCGAAA
AGATCGGCGAGCTGCTGGAGACCATCTTCGCTGTTGTTTTCAGCCACCGTCACCTGCAAGG
CATCCACCTGCAAGTTCCGTGTGAGGCTTTCCAATACCTGGTTAACAGCAGCAGCCAAATC
AGCGTTAAGGACAGCCAACTGCAAGTTTACAGCTTCAGCACCGACCTGAAGCTGGTTGACA
CCAACAAGGTTCAAGACTACTTCAAGTTCCTGCAAGAGTTCCCGCGTCTGACCCACGTGAG
CCAACAAGCTATCCCGGTTAGCGCTACCAACGCTGTTGAGAACCTGAACGTTCTGCTGAAG
AAGGTTAAGCACGCTAACCTGAACCTGGTTAGCATCCCGACCCAATTCAACTTCGACTTCT
ACTTCGTTAACCTGCAACACCTGAAGCTGGAGTTCGGCCTGGAGCCGAACATCCTGACCAA
GCAAAAGCTGGAGAACCTGCTGCTGAGCATCAAGCAAAGCAAGAACCTGAAGTTCCTGCGT
CTGAACTTCTACACCTACGTTGCTCAAGAGACCAGCCGTAAGCAAATCCTGAAGCAAGCTA
CCACCATCAAGAACCTGAAGAACAACAAGAACCAAGAGGAGACTCCGGAGACCAAGGACGA
GACCCCGAGCGAGAGCACCAGCGGCATGAAGTTCTTCGACCACCTGAGCGAGCTGACCGAG
CTGGAGGACTTCAGCGTTAACCTGCAAGCTACCCAAGAGATCTACGACAGCCTGCACAAGC
TGCTGATCCGTAGCACCAACCTGAAGAAGTTCAAGCTGAGCTACAAGTACGAGATGGAGAA
GAGCAAGATGGACACCTTCATCGATCTGAAGAACATCTACGAGACCCTGAACAACCTGAAG
CGTTGTAGCGTTAACATCAGCAACCCGCACGGCAACATCAGCTACGAGCTGACCAACAAGG
ACAGCACCTTCTACAAGTTCAAGCTGACCCTGAACCAAGAGCTGCAACACGCTAAGTACAC
CTTCAAGCAAAACGAATTCCAATTCAACAACGTTAAGAGCGCTAAGATCGAGAGCAGCAGC
CTGGAGAGCCTGGAGGACATCGACAGCCTGTGTAAGAGCATCGCCAGCTGTAAGAACCTGC
AAAACGTTAACATCATCGCTAGCCTGCTGTACCCGAACAACATCCAAAAGAACCCGTTCAA
CAAGCCGAACCTGCTGTTCTTCAAGCAATTCGAGCAACTGAAGAACCTGGAGAACGTTAGC
ATCAACTGTATCCTGGACCAACACATCCTGAACAGCATCAGCGAGTTCCTGGAGAAGAACA
AGAAGATCAAGGCTTTCATCCTGAAGCGTTACTACCTGCTGCAATACTACCTGGACTACAC
CAAGCTGTTCAAGACCCTGCAACAACTGCCGGAGCTGAACCAAGTTTACATCAACCAACAA
CTGGAGGAGCTGACCGTTAGCGAGGTTCACAAGCAAGTTTGGGAGAACCACAAGCAAAAGG
CCTTCTACGAGCCGCTGTGTGAGTTCATCAAGGAGAGCAGCCAAACCCTGCAACTGATCGA
CTTCGACCAAAACACCGTTAGCGACGACAGCATCAAGAAGATCCTGGAGAGCATCAGCGAG
AGCAAGTACCACCACTACCTGCGTCTGAACCCGAGCCAAAGCAGCAGCCTGATCAAGAGCG
AGAACGAGGAGATCCAAGAGCTGCTGAAGGCTTGTGACGAGAAGGGCGTTCTGGTTAAGGC
TTACTACAAGTTCCCGCTGTGTCTGCCGACCGGCACCTACTACGACTACAACAGCGACCGT
TGGTGAGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTCCCGGGAAGCTTGGGG

FIGURE 13